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Research Article

IN VITRO ANTIOXIDANT ACTIVITY OF *ANNONA MURICATA* LEAVES

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ABSTRACT

To check the antioxidant action of *Annona muricata* leaf extracts and to validate its therapeutic efficacy DPPH, ABTS, Hydrogen peroxide, Hydroxyl, Nitric oxide, reducing power assay, MTT dye reduction assay, and cell cycle analysis by flow cytometry were performed. DPPH assay revealed that methanol extract showed 82.05% scavenging activity at 500µg concentration. In ABTS radical scavenging activity, ethyl acetate extract exhibited 91.03% scavenging activity at 500µg concentration. Ethanol extract actively exhibit the highest scavenging action against H_2O_2 radicals; the scavenging action was found to be 94.10% at 500µg concentration. Hydroxyl scavenging assay revealed that aqueous extract showed a 93.50% scavenging activity at 500µg concentration. Nitric oxide radical scavenging assay revealed that methanol extract showed 94.53% scavenging activity at 500µg concentration. Reducing power ability results showed that aqueous extracts were able to mitigate Fe^{3+} ions in a dose-dependent manner, and maximum action was seen to be at 500µg concentration. MTT assay reveals that aqueous extract showed better activity, and the IC_{50} value was found to be at 195.42µg concentration, the IC_{50} values of methanol and ethanol extracts were 318µg and 398.40µg, respectively. Cell cycle analysis reveals that aqueous, methanol, and ethanol extract-treated group was arrested at S phase. The results suggest that *Annona muricata* leaves extract is an encouraging applicant for the treatment of oxidant related diseases and deserves additional research as an alternative to conventional drugs.

Keywords: *Annona muricata*, *In vitro*, Radical scavenging, Antioxidant activity, Ovarian cancer

1. INTRODUCTION

Ovarian cancer brings about critical mortality in women and is one of the utmost predominant sorts of malignant gynecological growth everywhere throughout the world, regularly analyzed at cutting edge stages and at present utilized anticancer drugs produces various adverse effects [1].

Particles or atoms with unpaired electrons are characterized as free radicals and are accordingly known as Reactive Oxygen Species, it includes superoxide, nitric oxide, hydroxyl, and hydrogen peroxide radical [2]. The macromolecules, for example, nucleic acids, lipids, and proteins usually assaulted by free radicals and prompts cellular death and homeostatic disruption [3]. Antioxidants serve as free radical scavengers by shielding the living substance from the staggering impacts of unusual ROS production. An absence of harmony between the antioxidant system and free radicals can prompt different pathological conditions [4]. In current years, antioxidants from natural sources have a prevalent role in the pharmaceutical industry because of their nutritional and therapeutic values [5]. Drugs extracted from natural herbal sources are described as cost-effective and safe for consumption [6]; natural herbal products were a wealthy origin of small molecule drug discovery and compounds [7].

One method for treating malignancy is to pick up control or potentially end the uncontrolled development of cancer cells. Utilizing the cell's system for death is a profoundly successful strategy. Furthermore, focusing on apoptosis is the best, non-surgical treatment [8]. Existing apoptosis-inducing chemotherapies have adverse side effects for patients. The exploration for less toxic drugs is therefore a necessity and natural herbal products are required to support in the evolution of drugs that modulate apoptosis [9].

Annona muricata L. belongs to the family Annonaceae is regularly known as Graviola or soursop. The phyto-constituents that is naturally present in the plant shows disease preventive properties. Annonaceous aceto-genins, lactones, isoquinoline, tannins, alkaloids, and coumarins are few bioactive compounds present in the

Annona muricata leaves [10].

The Kingdom Plantae has contributed many effective medicines for the treatment of peculiar diseases, as well as malignant growth and it remains to be an origin of various new potential therapeutic small molecules. For instance, secondary metabolites present in the Annonaceae family known as annonaceous acetogenins (AAs), which are historically occupied plants for the treatment of malignant growth and many other diseases [9]. Therefore, research was formulated to examine the capacity of *Annona muricata* leaf extract for its antioxidant action.

2. MATERIAL AND METHODS

2.1. Chemicals

Solutes and solvents of analytical grade were used in the assays carried in the present study.

2.2. Culturing of ovcar-3 cell line

Cell lines (OVCAR-3) were maintained as per the protocol given by NCCS, Pune, India. The medium used for subculture is RPMI 1640 medium supplemented with 10% Fetal Bovine Serum, 0.5% penicillin-streptomycin, and incubated at 37°C in a CO_2 incubator.

2.3. Authentication of plant material

The leaves of A. *muricata* collected from local areas of Coimbatore, Tamilnadu was authenticated by the Head, Botanical Survey of India, Coimbatore (BSI/SRC/5/23/2019/Tech/192).

2.4. Preparation of sample

Ten (10)g of powdered leaf sample was weighed and extracted (100ml) by Soxhlet extraction in different solvents with varying polarity petroleum ether (non-polar), benzene(non-polar), ethyl acetate (polar), chloroform (non-polar), methanol (polar), ethanol (polar), water (polar), acetone. After extraction, the extracts were allowed to evaporate in a temperature controlled water bath at 60°C. The concentrated extracts were then dissolved in DMSO to attain a final concentration of 1mg/ml and used for the assays performed in the present study.

2.5. Evaluation of free radical scavenging activity of *Annona muricata* leaf extracts

2.5.1. DPPH radical scavenging assay

Based on the scavenging activity of the stable DPPH-free radical, the antiradical action of the *Annona* extracts was calculated as stated by Brand-Williams *et al*. [11] with minor modifications. A 0.5ml of plant extract solution of varying concentrations (25, 50, 100, 200, 300, 400 and 500µg/ml) was added in methanol with 0.5ml of 0.1mM of DPPH. A corresponding blank sample was prepared. The reaction containing 0.5ml of methanol and 0.5ml of DPPH solution served as control. The experiment was accomplished in triplicate, and the decrease in absorbance was measured at 520 nm, after half an hour of dark incubation using a UV-Vis spectrophotometer. The % scavenging was determined as follows: % scavenging = $[(A_{control} - A_{sample})/ (A_{control})] \times 100$.

2.5.2. ABTS radical scavenging assay

Radical quenching potential of the extracts were determined using ABTS radical cation decolorization test, as stated by Shirwaikar *et al*. [12]. A reaction with ABTS solution (7mM) with (2.45mM) ammonium persulfate in dark at room temperature for 12 hours was done for the ABTS radical cation to be performed under laboratory conditions. 0.7ml of varying concentration plant extract solution (25, 50, 100, 200, 300, 400 and 500µg/ml) combined with 0.3ml of ABTS. The absorbance was read at 745 nm and the % ABTS radical scavenging was determined as follows: % scavenging = $[(A_{control} - A_{sample}) / (A_{control})] \times 100$.

2.5.3. Hydrogen peroxide scavenging activity

According to Ruch *et al*. [13], the ability of *Annona* extracts to scavenge H_2O_2 was determined. For this, 43mM solution of H_2O_2 in 0.1M phosphate buffer (pH 7.4) was prepared. 2.4ml of plant extract solution of varying concentrations (25, 50, 100, 200, 300, 400 and 500µg/ml) was mixed to 0.6ml of 43mM H_2O_2 solution. Sodium phosphate buffer without H_2O_2 was served as blank. The O.D. units was measured at 230nm and the % scavenging action by the extract was determined using the formula, % scavenging = $[(A_{control} - A_{sample}) / (A_{control})] \times 100$.

2.5.4. Hydroxyl radical scavenging activity

The scavenging activity of *Annona muricata* leaf extracts against hydroxyl radicals was also measured in the present study, based on the Klein *et al*. method [14]. *Annona* leaf extract with varying concentrations (25, 50,100, 200, 300, 400 and 500µg/ml) was used in the assay. The following reagents were sequentially added with1.0ml of iron-EDTA solution, 0.5ml of 0.018 %

EDTA solution and 1.0ml of DMSO (0.85% DMSO (v/v) in 0.1 M phosphate buffer, pH 7.4). The reaction was started by mixing 0.5ml (0.22 %) of ascorbic acid, and incubated in a temperature controlled water bath at 90°C for about 15min. The scavenging action was stopped after incubation by adding 1.0ml (17.5% w/v) ice-cold TCA. Added 3ml of Nash reagent and incubated for 15min at room temperature. The control tubes were also performed without the addition of the sample. The intensity of the color developed was measured at a wavelength of 412nm against reagent blank. The quenching ability of *Annona* extracts against hydroxyl radical was obtained as follows: % scavenging $= [(A_{control} - A_{sample}) / (A_{control})] \times 100$

2.5.5. Nitricoxide radical scavenging assay

Nitric oxide radicals were generated using sodium nitroprusside and measured by Griess reaction, according to the experimental procedure of Annie *et al*. [15]. 5mM sodium nitroprusside in phosphate buffer saline solution (0.025M, pH: 7.4) was allowed to incubate with varying concentration of *Annona* plant extracts (25, 50, 100, 200, 300, 400 and 500µg/ml) dissolved in standard phosphate buffered saline solution (0.025M, pH: 7.4) and incubated at 25°C for 5hr. Control tubes without sample but with equivalent amounts of buffer were organized in the same manner. After the incubation period of 5 hours, 0.5ml of the incubated mixture was taken out and diluted with 0.5ml of Griess reagent (1% sulphanilamide, 2% ortho-phosphoric acid, and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance was taken at 546nm and the percentage scavenging was determined as follows: % scavenging $= [(A_{control} - A_{sample}) / (A_{control})] \times 100$

2.5.6. Reducing power assay

The reducing ability of the *Annona* extracts was accomplished as defined by Yildrenim *et al*. [16].The various concentration of extracts (25, 50, 100, 200, 300, 400 and 500µg/ml) were mixed with equal volume (2.5ml) of 1% potassium ferricyanide and sodium phosphate buffer (0.2M, pH 6.6). The solution was allowed to react for 30 minutes at 50°C. To the reactants, 2.5ml of 10% trichloroacetic acid was poured and the solution mixture was centrifuged at 2000g for 10 minutes. The uppermost layer (2.5ml) was added with 2.5ml of deionized water and 0.5ml of ferric chloride and the O.D units were measured spectro-photometrically at a wavelength of 700nm.

2.6. Assessment of anticancer activity of *Annona muricata* leaf extracts

Based on the results of radical scavenging assays, aqueous, methanol, and ethanol leaf extracts were chosen for anticancer studies.

2.6.1. MTT dye reduction assay

As reported by the method, Igarashi et al. [17], MTT dye reduction assay was performed. The experiment was carried out using different concentrations of leaf extracts ranging from 100, 200, 300, 400, and 500µg respectively. 100µl of treated ovarian cell lines were treated with 50µl of MTT at 37°C for 3 hours. After 3 hours of incubation, 200µl of PBS was layered over all the treated wells in the microtiter plate and aspirated cautiously to take out surplus MTT.200µl of acid-propanol was further added to solubilize and left overnight in the dark. In a micro titer plate reader, absorbance was read at 650nm. Optical density obtained for the control cells were fixed to be 100% viable and the percent viability of the cells in various treatment groups were calculated using the formula: % Viability = [(Control OD - Sample OD)/ (Control OD)] × 100

2.6.2. Cell cycle analysis by flow cytometry

Cell cycle analysis was done using flow cytometry as per the protocol described by Krishan [18]. Cells treated with the IC_{50} values of plant extracts for 24 hours, were trypsinized and centrifuged. Then the cells were incubated with 1ml of the prepared mixture of Propidium iodide reagent and were stained for about half an hour at room temperature in dark. After the cells were incubated for the specified time period, the cells were examined for the populations of sub-G0, G0/G1, S, and G2/M phases by flow cytometry. The results were interpreted using FACSuite software.

2.7. Data analysis

The results of individual series of experiments were performed in triplicates and expressed the mean ± standard deviation using Microsoft Excel 2010 and to determine the significance of the EC_{50} values of standard and treatment groups, IC_{50} values of different treatment groups, statistical analysis was performed by one-way ANOVA followed by post hoc Tukey's multiple comparison tests using Graph pad prism 8.4.1 (Trial version). The $P<0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Free radical scavenging activity of *Annona muricata* leaves

3.1.1. DPPH radical scavenging assay

The DPPH results revealed that 8 different extracts were capable of scavenging DPPH radicals, among the extracts tested, methanol, water, and ethanol leaf extracts displayed greater % scavenging activity of 82.05±0.85, 79.51±0.34, 79.34±0.41 at 500µg concentration and their EC_{50} values were found to be 75.92±0.25, 118.76±0.95, 121.40±0.36 µg/ml respectively (Fig. 1). Phenolic compounds were frequently found in plants, and several biological activities have been documented, including potent antioxidants and free radical scavengers apart from the primary function of defense [19]. Polyphenols can work by various radical quenching mechanisms, including metal scavengers, passing electrons, or contributing hydrogen ions; specifically elevated level molecular weight phenolic components have a greater capacity to neutralize free radicals [20, 21]. Thus the results of DPPH assay could be due to the presence of polyphenols and other phytoconstituents.

3.1.2. ABTS radical scavenging assay

In ABTS radical scavenging activity, the results revealed that all the extracts with different concentrations tested possess scavenging activity. Among the extracts tested, the ethyl acetate, ethanol and water leaves extract of A. *muricata* rendered maximum % scavenging activity of 91.03±0.19, 75.31±0.13, 73.39±0.19 at 500µg concentration towards ABTS radical, and their EC_{50} values were found to be 188.55±0.71, 54.73±0.67, 154.46±1.26µg/ml (Fig. 2). Tannins, excessive molecular weight phenolic components have increased potential to quench ABTS free radicals. Thus the results of ABTS radical scavenging assay revealed that ethyl acetate, ethanol and aqueous leaf extracts of *A. muricata* has possess good radical scavenging potential and this could be attributable to the increased levels of polyphenols, elevated molecular weight phenolics of *A. muricata* leaves and due to the transfer of electrons between the antioxidant compounds and ABTS radical.

3.1.3. Hydrogen peroxide radical scavenging assay

In the present study, 8 different extracts with varying concentrations were subjected to oxidative stress by adding H_2O_2 to a considerable extent. All the extracts analyzed possess substantial scavenging activity. Among the extracts tested, ethanol, ethyl acetate, and petroleum ether showed increased activity with increasing concentration and their maximum % scavenging activity was found to be 94.10±0.32, 90.45±0.23, 88.63±0.28, EC_{50} values were 221.00±0.88, 91.77±0.31, 370.89±1.74µg/ml respectively (Fig. 3). The research showed those phytochemical components that have been documented to have multiple therapeutic impacts, including antioxidant action, such as flavonoids and other phenolic components [22]. Extracts can be related to the scavenging of H_2O_2 by their phenolic compounds which can contribute electrons to H_2O_2, thereby neutralising it to water [23].The findings reveal that tested extracts had a strong scavenging action of H_2O_2, which might be due to the antioxidant components and mixtures. Since good electron donors are the antioxidant components and mixtures present in the tested extracts, they can accelerate the conversion of H_2O_2 to H_2O [24]. Similar results were noticed in the present research study, in compliance with these cited literatures, the result of current investigation shows that the flavonoids and phenolics existing in the leaves extract might be accountable for scavenging activity.

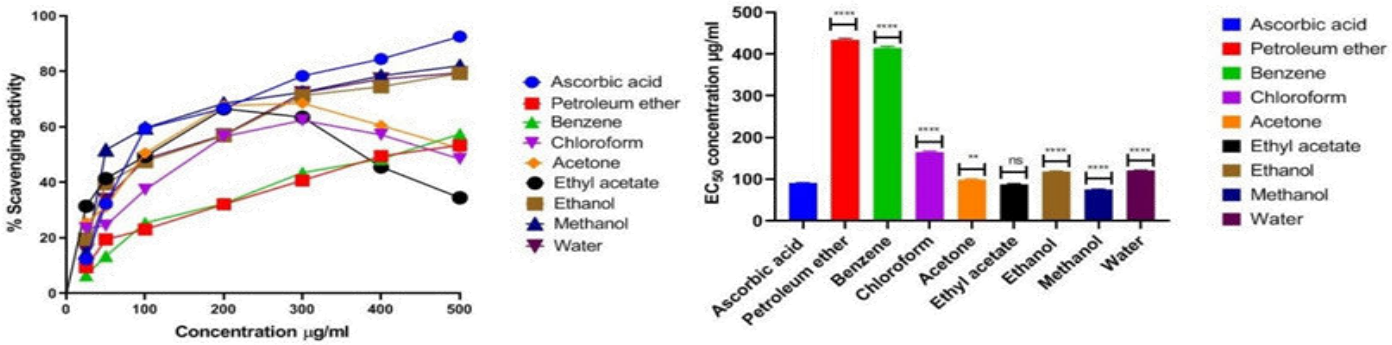

Fig. 1: Scavenging action of *Annona muricata* extracts against DPPH

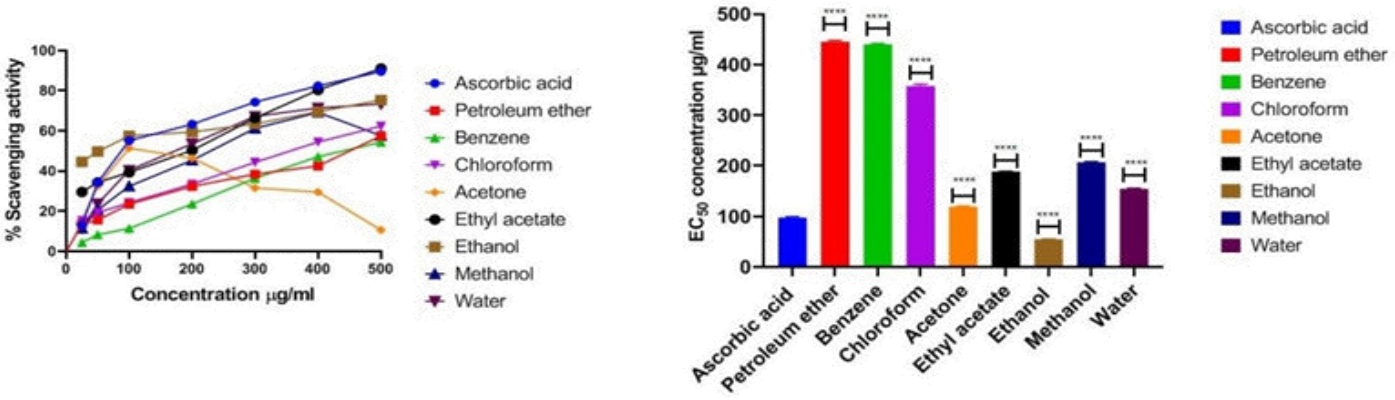

Fig. 2: Scavenging action of *Annona muricata* extracts against ABTS

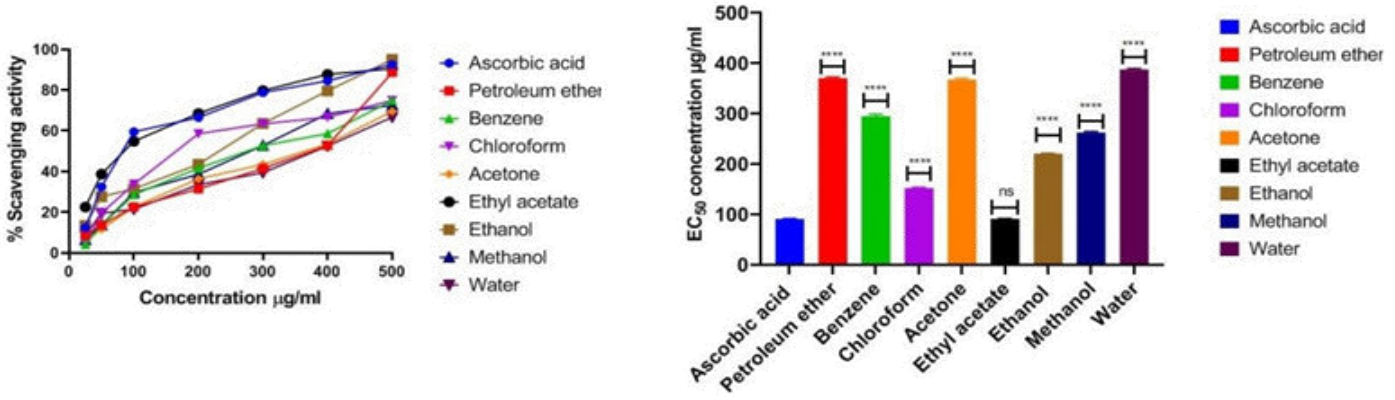

Fig. 3: Scavenging action of *Annona muricata* extracts against Hydrogen peroxide

3.1.4. Hydroxyl radical scavenging assay

The results revealed that all the extracts with different concentrations tested possess hydroxyl radical quenching ability. Among the extracts tested, water, ethyl acetate, and ethanol extract, of A. *muricata*, rendered maximum % scavenging activity of 93.50±0.14, 84.40±0.22, 81.79±0.19 towards hydroxyl radical, in a dose-dependent manner and their EC_{50} values were 178.00±0.95, 101.49±1.38, 146.94± 1.23µg/ml respectively (Fig. 4). polyphenols, like flavonoids that have solubility in less polar solvents, may be capable of chelating iron and copper metal ions as the enormous quantity of hydroxyl groups (OH) in their compound structure, which is prone to present chelating capacity [25]. The findings of the current investigation showed that the flavonoids and phenolics existing in the leaves extract might be accountable for the scavenging effect of the muricata plants, following this cited literature, similar results were observed in the present research.

3.1.5. Nitric oxide radical scavenging assay

The eight different solvent extracts were tested for their scavenging potential against NO generation *in vitro* in the present research study. All the solvent extracts possessed considerable scavenging activity against radical generation. Among the extracts tested, methanol, chloroform, and benzene leave extract showed maximum % scavenging activity of 94.53±0.37, 79.76±0.31, 78.39±0.36 at a concentration of 500 µg and their EC_{50} values were 141.13±1.38, 245.87±3.85, 192.03±4.07 µg/ml respectively (Fig. 5). Sasi Kumar *et al.*, (2014) [24] reported that all the tested *K. foetidissima* extracts successfully lowered the production of nitric oxide from sodium nitroprusside. Methanolic extract displayed excessive nitric oxide radical inhibition in contrast with other extracts. Nitric oxide scavenging assay was carried out with various concentrations of *Baccaurearami flora* fruit juice (BRJ). The results showed that NO radical formed was significantly inhibited by the *Baccaureasami flora* fruit

juice in a dose dependent manner and reported that the large content of phenolic and flavonoid components noticed in BRJ could have performed a considerable part in free radical scavenging activities [26]. In compliance with these cited literatures, the result of current investigation shows that the phenolics, flavonoids and antioxidants existing in the leaves extract might be accountable for scavenging activity by inhibiting the formation of nitrite radical by competing with oxygen and oxides of nitrogen in the reaction mixture.

3.1.6. Reducing power assay

The reducing capacities of 8 different solvent extracts were measured by their capability to transform Fe^{3+} to Fe^{2+} at various concentrations. The results revealed that water; methanol and ethanol leaves extract were able to reduce Fe^{3+} ions, the action increased with a hike in the concentration of the extracts which indicates the dose-dependent response of the extracts and maximum activity was found to be at a concentration of 500µg (Fig. 6). Various therapeutic effects have been reported including antioxidant activity in phytochemical components such as flavonoids and other phenolic compounds [22]. There is a reduction in ferric ions, due to the efficiency or impact of all of these compounds existing in the extracts. Abundant research studies have demonstrated that the bulk of plant food's antioxidant production is from phenolic products such as anthocyanins, flavonoids, flavones, isoflavones, catechins, and isocatechins instead vitamins C and E and β-carotene [27-29]; and are trusted because of their redox properties [30], and that play an important aspect in absorbing and destroying free radicals, triplet oxygen and quenching singlet, chelating metal catalysts, decomposing peroxides and enhancing antioxidant enzyme activities [31].

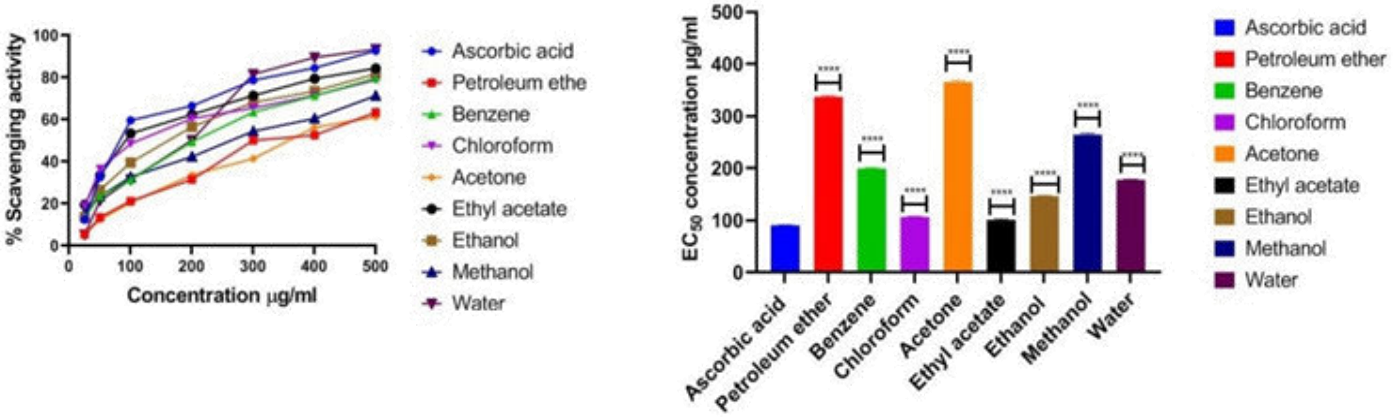

Fig. 4: Scavenging action of *Annona muricata* extracts against Hydroxyl radical

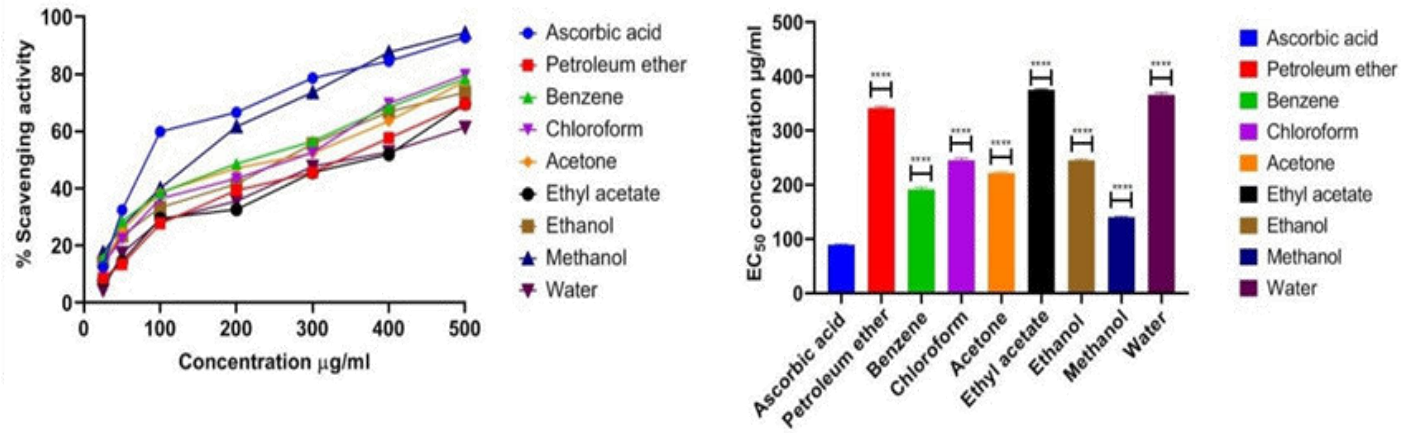

Fig.5: Scavenging action of *Annona muricata* extracts against Nitric oxide

3.2. Anticancer activity of *Annona muricata* leaf extracts

3.2.1. MTT dye reduction assay

The results reveal that aqueous, methanol, and ethanol leaf extract of *Annona muricata*, was capable of inducing cell death (Fig. 7). When the OVCAR-3 cell line was treated with increasing concentration, the viability was found to be decreased. Among the groups tested aqueous extract showed better activity followed by methanol and ethanol extracts and their IC_{50} values were found to be 318±14.24, 195.42±5.88, 318±14.24µg /ml respectively (Fig. 7). Free radical inhibitory potential of Graviola aqueous extract was found to be more, which might be due to the great antioxidant status when compared to ethanolic extract of Graviola on EACC, MDA, and SKBR3 cell lines [32]. *Annona muricata* methanolic leaf extract demonstrated maximum anticancer activity on the MCF-7 cell line [33]. *Annona muricata* ethanolic leaves extract on HepG2 cells has been capable of causing cell death in a dose-dependent manner [34]. The *in vitro* anticancer activity of *Annona muricata* leaves on the OVCAR-3 cell line may be attributable to the existence of the numerous bioactive compounds which are very well evidenced by the results of antioxidant assays.

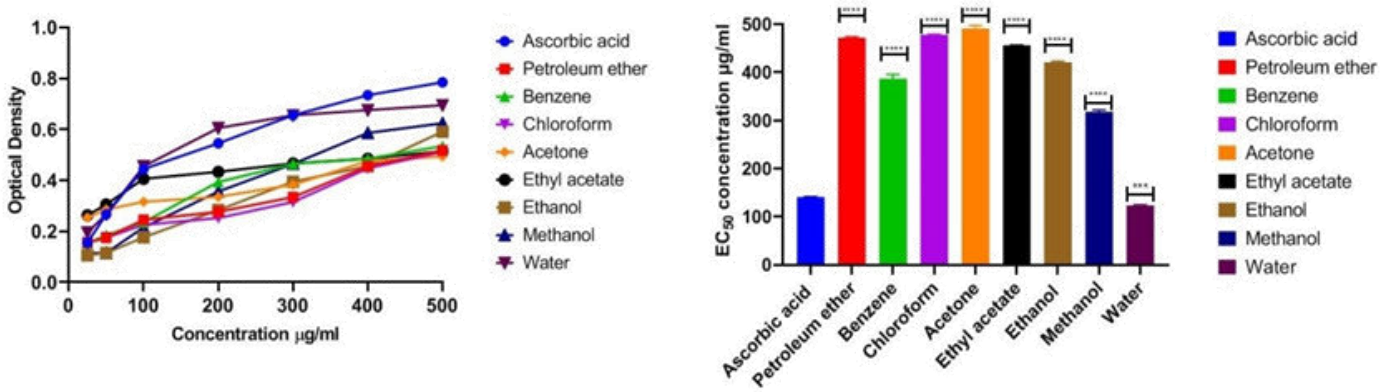

Fig. 6: Reducing power activity of *Annona muricata* leaf extracts

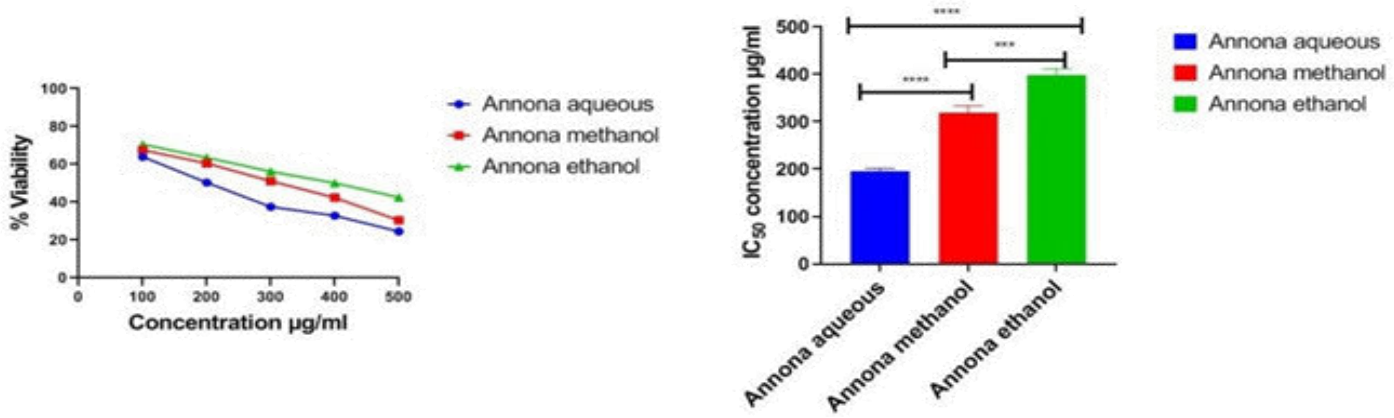

Fig. 7: Graphical representation of Cytotoxic effect of *Annona muricata* leaf extracts by MTT assay and IC_{50} values of water, methanol and ethanol leaf extracts of *Annona muricata*

3.2.2. Cell cycle analysis by flow cytometry

The results showed that, in the untreated OVCAR-3 cells, the cells were evenly distributed in all the phases of the cell cycle, which indicated that there was no cell cycle arrest. In case of *Annona muricata* aqueous, methanol, ethanol leaf extracts treated OVCAR-3 cells, were arrested at S phase, and methanol extract-treated group showed a higher proportion of cells were arrested at S phase (2.23%), indicating that cells were arrested in synthesis phase of all treated groups (Fig. 8, 9).The blockade of the cell cycle is seen as an important action for the production of innovative cancer therapies [35].

DNA replication initiation with drug interference can forever arrest the cancer cells at the S phase. Arrest in the S phase includes active suppression by suppressor transactivation, phosphorylation of essential replication control elements, and direct interaction of suppressors to replication machinery [36]. Cell cycle analysis exhibited that *A. atroviolaceum* flower treated MCF-7 cells induced an S and G2/M phase cell cycle arrest with the subsequent reduction in the G1 phase at 24 and 48 h of treatment. This confirmed that *A. atroviolaceum* flower inhibited DNA synthetic phase in the cell cycle and induced a block at the S and G2/M phase boundary at 24 and 48h [37]. Cucurbitacin B caused S phase arrest in the cell cycle when exposed to BEL-7402 cells [38]. Our results were consistent with the data published by other groups; all the three treatment groups showed a cell cycle arrest in the S phase, showing *Annona muricata* plant extracts is capable of inhibiting DNA synthesis.

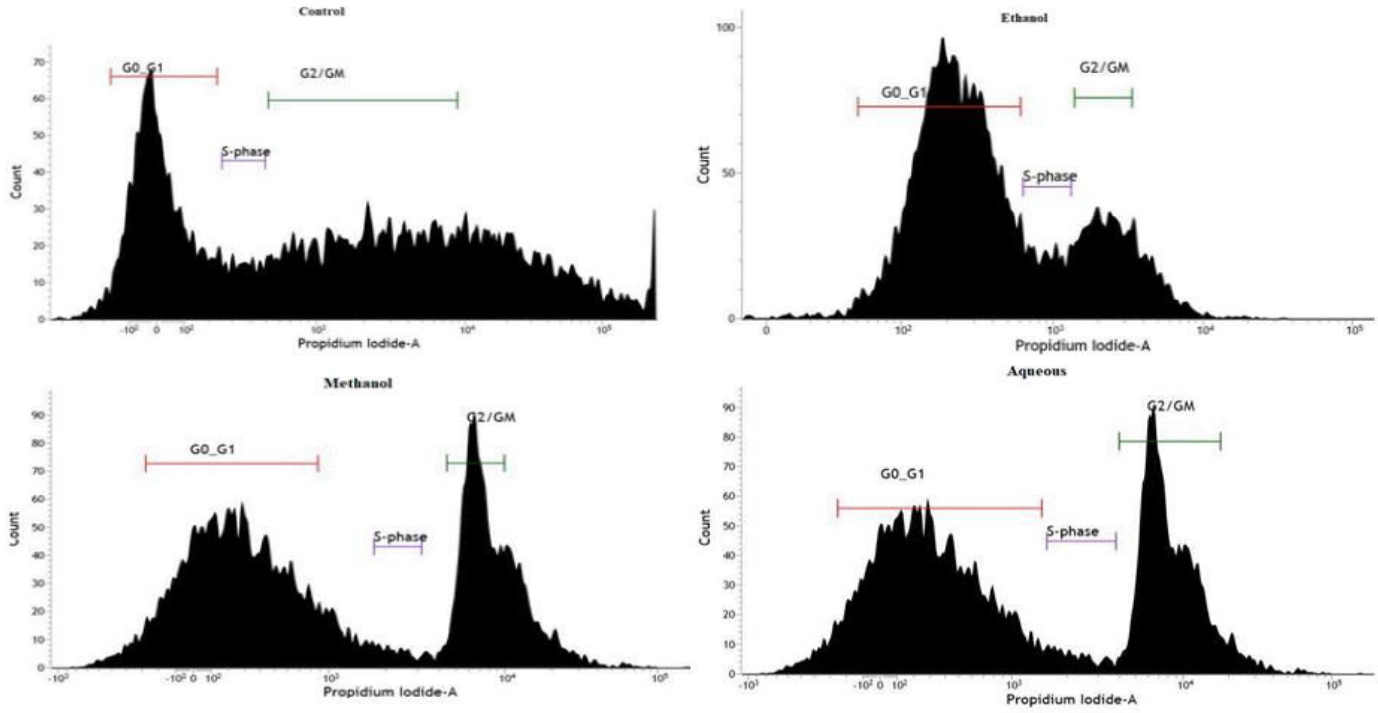

Fig. 8: Effect of cell cycle distribution of OVCAR-3 cells exposed to a) untreated group (Control), b) ethanol extract, c)methanol extract, andd)aqueous extract

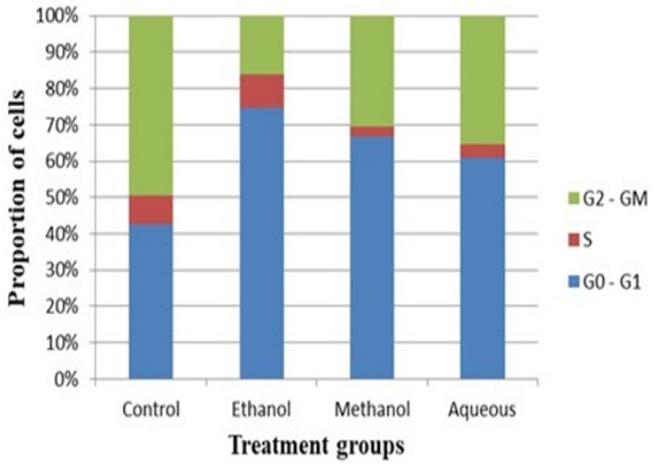

Fig. 9: Graphical representation of the distribution of cells in various phases of cell cycle

4. CONCLUSION

Thus it can be concluded that the *Annona muricata* leaf extracts of differing polarity could readily scavenge the team of radicals including DPPH, ABTS, hydroxyl radicals, hydrogen peroxide and nitric oxide, indicating the potency of *Annona muricata* leaf extracts against radical-mediated diseases and disorders. The reducing ability of the solvent extracts analyzed evidenced the existence of phytoconstituents and antioxidant principles in the *Annona muricata* leaf extracts which might be responsible for all the radical quenching ability thereby preventing several many diseases and disorders associated with oxidants and free radicals. The overall antioxidant and anticancer activity of the leaves extract might be attributed due to the existence of secondary metabolites such as flavonoids and phenols. Our

observation might be used in the ethnopharmacological approach for exploiting the possible therapeutic agents in treating and preventing various diseases caused by free radicals. Further, structural and functional elucidation of these *Annona muricata* leaf extracts has to be explored.

5. ACKNOWLEDGEMENT

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Conflicts of interest - Nil

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