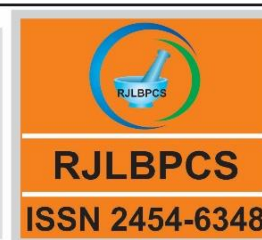

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IN VITRO ANTIPROLIFERATIVE AND FREE RADICAL SCAVENGING ACTIVITIES IN PEELS OF CITRUS FRUITS

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ABSTRACT: The present study was contemplated to evaluate the *in vitro* antiproliferative and antioxidant activities in ethanolic extracts of citrus fruits peels *viz,* Lime (*Citrus aurantifolia*), Orange (*Citrus reticulata*), Sour Orange (*Citrus aurantium*), Pomello (*Citrus grandis*) and Citron (*Citrus medica*) on MCF-7 breast cancer cell line and U-87MG glioblastoma cell line. The peels of the fruits were separated and subjected to cold extraction using 70% alcohol. The extracts obtained were subjected for qualitative phytochemical analysis to detect the presence of different phytoconstituents and the antiproliferative activity of the extracts was investigated *in vitro* through MTT assay. Further the antioxidant activity of the extracts was assessed by using ABTS and hydroxyl radical scavenging methods. The results of qualitative phytochemical analysis revealed the presence of several bioactive compounds such as polyphenols, flavonoids, terpenoids, steroids, glycosides, saponins and alkaloids. The peels of all five citrus fruits registered notable increase in the cell proliferation inhibition and antioxidant activities in a dose dependent manner. The results of anti-proliferative activities were expressed in terms of IC_{50} where pomello peel extract showed considerable cytotoxicity and cell proliferation inhibition in MCF-7 with an IC_{50} value of 872±0.43 μg/mL, followed by lime (978±0.55μg/mL), sour orange (1920±0.42μg/mL), orange (1965±0.83 μg/mL) and citron (2000±0.49 μg/mL). While for U-87MG orange peel extract showed significant decrease in cell proliferation with an IC_{50} value of 778±0.768 μg/mL whereas sour orange, lime, citron and pomello exhibited 870±0.57, 1076±0.45, 1116±0.66 and 1647±0.42 μg/mL IC_{50} respectively. The findings from study thus indicated that citrus fruit peels possessed immense potential as therapeutic sources in treatment of cancer and support their health promoting claims of plethora of investigations.

KEYWORDS: Citrus peel, anti-proliferative, MTT, antioxidant, ABTS, Hydroxyl.

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1. INTRODUCTION

Cancer is the life threatening disorder characterized by the abnormal proliferation of cells that invade the adjoining tissues and cause the destruction of those tissues. It is one of the most significant and leading cause of human death all over the world [1]. Several chemotherapeutic agents are being used for the management of cancer but the problem of selective toxicity and severe side effects still exist. An alternative solution for the harmful effects of synthetic agents is the use of plant derived natural products which provide an outstanding contribution to modern therapeutics due to their cytotoxic and cancer preventive effects [2,3]. Drug discovery from medicinal plants has played an important role in the treatment of cancer and indeed, over the last half century most of the plant secondary metabolites and their derivatives have been used towards combating cancer [4-6]. Incidentally rich biodiversity and traditional medical knowledge had provided valuable compounds for cancer chemotherapy [7,8]. Consequently, traditional medicinal plants can serve as potential sources in the development of new, more effective anticancer agents for future therapy. Oxidative stress is one of the crucial biological responses induced by tumor promoters. Most of mutagenic and carcinogenic agents reveal their destructive effects through free radicals including reactive oxygen's species (ROS). ROS have a role in etiology of diseases such as cancer, cardio cellular, neural problems and senescence. Suppression of the oxidative stress induced by the generation of excessive amounts ROS is now widely ascribed as a new paradigm for most cancer chemoprevention [9-11]. So daily consumption of antioxidants enhances immunity of the body against free radical production and serves as anticancer agent. Cytotoxicity screening models provide imperative preliminary data to select plant extracts with significant antineoplastic potentialities [12]. Various methods are in use to determine the effect of a drug on cells propagated *in vitro.* They range from simple assays that measure cell viability after drug exposure, *i.e.* dye exclusion that measures membrane integrity and effect of the drug on cell growth, to different assays that measure cell viability indirectly, by assessing the capability of the cell to reduce compounds such as MTT, XTT, MTS, SRB, and alamar blue or to generate ATP [13]. The plant sources of India are likely to provide effective anti-cancer agents. These activities have been co-related to the presence of certain phytochemical substances. Epidemiological studies have constantly linked abundant consumption of fruits and vegetables lower the risk of developing at least 20% of all cancer instances [14,15]. Some of the fruits and vegetables are considered as the main anticancer foods, because of their abundant antioxidants such

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as phenols, vitamin C, vitamin E, beta-carotene and lipotene [16]. Citrus fruits are of specific interest for cancer prevention, since they are rich in vitamin C, flavanones (a class of flavonoids), and other compounds with antioxidant, antimutagenic, and anti-proliferative properties [17-19]. However the studies related to anticancer properties involving different cell lines is rather sporadic and moreover such attempt on Indian context is strangely scanty. Therefore the current comparative study was aimed to evaluate the *in vitro* antiproliferative and antioxidant effects of ethanolic extracts obtained from the peels of some commonly grown yet unexplored citrus fruits of South India *viz.* Lime, Orange, Sour Orange, Pomello and Citron on breast cancer cell line (MCF-7) and glioblastoma cell line (U-87MG) using MTT assay while ABTS (2,2-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid) and Hydroxyl radical scavenging activities have been used to assess their antioxidant property.

2. MATERIALS AND METHODS

Collection of Plant Materials

The Citrus fruits were procured from a local market of Shimoga, Karnataka. Which were authenticated by the Taxonomist, Department of Botany, Sahyadri Science College, Shimoga. The fruits selected comprise Lime (*Citrus aurantifolia*), Orange (*Citrus reticulata*), Sour Orange (*Citrus aurantium*), Pomello (*Citrus grandis*) and Citron (*Citrus medica*). After collection, fruits were washed under running tap water followed by washing with distilled water to remove the surface debris. Then the peels of the fruits have been separated and were further subjected for extraction procedures.

Extraction

Exactly 1000g of the separated peel from each fruit category was subjected to extraction using 70% ethanol as per the method described by Pallavi *et al* 2017 [20].

Qualitative Phytochemical Analysis

The phytochemical screening was performed for testing the different chemical groups present in ethanolic extracts of peels from all citrus fruits [21-22].

In Vitro Anti-Proliferative Activity

Cell Lines and Culture

Breast cancer MCF-7 and Glioblastoma U-87MG cell lines of human origin were obtained from National Centre for Cell Sciences, Pune (NCCS). MCF-7 is a human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors. It is derived from the pleural effusion of a 69-year-old Caucasian metastatic breast cancer in 1970. MCF-7 cells are useful for *in vitro* breast studies because they retained several ideal characteristics particular to mammary epithelium, such as the processing of estrogen, in the form of estradiol, via estrogen receptors (ER) in the cell cytoplasm [23].U87 is a human primary glioblastoma cell line that is commonly used in brain cancer research. The U87 cell line has an epithelial morphology and was obtained from a 44-year-old female patient in 1996 at Uppsala University [24]. The cells were cultured in Dulbecco's

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Modified Eagle's Medium (DMEM) supplied with 10% heat-inactivated FBS (Invitrogen, USA) and 1% Penicillin-Streptomycin (Sigma-Aldrich, USA) and 0.37% sodium bi-carbonate (Sigma-Aldrich, USA) was used at 37°C in a 5% CO_2 enriched humidified incubator (Thermo scientific, USA) with 98% humidity.

Anti-Proliferative Activity by MTT Assay

Antiproliferative activity of the extracts against the selected cell lines was tested using the microtitration colorimetric method of MTT reduction [25] with minor modifications as required. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is used to determine cell viability in cell proliferation and cytotoxicity assays. The cells were trypsinized and aspirated into a 15ml centrifuge tube. Cell pellet was obtained by centrifugation at 300 x g. The cell count was adjusted using DMEM HG medium for MCF-7 and MEM HG medium for U87MG cell lines, such that 200µl of suspension contained approximately 10,000 cells. To each well of the 96 well microtitre plate, 200µl of the cell suspension was added and the plate was incubated at 37ºC and 5% CO_2 atmosphere for 24 h. After 24 h, the spent medium was aspirated. 200µl of different concentrations of extracts (250, 500, 1000, 1500 and 2000 µg/ml from stock) were added to the respective wells. The plate was then incubated at 37ºC and 5% CO_2 atmosphere for 48 h. The plate was removed from the incubator and the extract containing media was aspirated. 200µl of medium containing 10% MTT reagent was then added to each well to get a final concentration of 5mg/ml and the plate was incubated at 37ºC and 5% CO_2 atmosphere for 4 h and then the medium was aspirated and replaced with 100 µl DMSO to dissolve the formazan crystals formed. The cells were incubated for 10 min and the absorbance of each well was read using micro-ELISA reader (Robonics, India) at 570nm. The results were produced from independent experiments, and each experiment was performed in triplicate for each cell line. The concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. The effect of the samples on the proliferation of cell lines was expressed as the % cell viability, using the following formula:

% cell viability = Absorbance of treated cells / Absorbance of control cells × 100%.

% cell inhibition = 100 − % cell survival

A dose response curves were plotted to enable the calculation of the concentration test sample required to inhibit cell growth by 50% (IC_{50}) for each cell line.

Evaluation of*In Vitro* Antioxidant Activities

2,2-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) radical Scavenging activity

ABTS free radical scavenging assay was measured by employing the method of Auddy *et al* 2003 with some modifications [26]. The stock solutions included 7mM ABTS solution and 2.4mM ammonium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 16 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS with 10 ml PBS to obtain an absorbance of

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≥1.0 units at 734 nm using a spectrophotometer. A fresh solution of ABTS was prepared for each assay. Different concentrations of peel extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using a spectrophotometer. All experiments were performed in triplicates. The ABTS scavenging ability of the extract was compared with that of standard ascorbic acid and percentage inhibition was calculated using the following formula. The results are expressed as EC_{50}, which is the amount of antioxidants necessary to decrease the initial concentration by 50%.

$$\text{Percentage Effect (E \%)} = (A_0 - A_1)/A_0 \times 100$$

Where,

A_0 is the absorbance of the control (without test samples) and

A_1 is the absorbance of test samples.

Hydroxyl Radical Scavenging Activity

Hydroxyl radicals were generated by a $Fe3^+$-ascorbate-EDTA-H_2O_2 system (Fenton reaction) and the scavenging capacity towards the hydroxyl radicals was measured by using deoxyribose method [27]. The reaction mixture contained 2-deoxy-2-ribose (2.8 mM), phosphate buffer (0.1 mM, pH 7.4), ferric chloride (20 µM), EDTA (100 µM), hydrogen peroxide (500 µM), ascorbic acid (100 µM) and various concentrations of the peel extracts in a final volume of 1 ml. The mixture was incubated for 1 h at 37 °C. After the incubation an aliquot of the reaction mixture (0.8 ml) was added to 2.8% TCA solution (1.5 ml), followed by TBA solution (1% in 50 mM sodium hydroxide, 1 ml) and sodium dodecyl sulphate (0.2ml). The mixture was then heated (20 min at 90 °C) to develop the colour. After cooling, the absorbance was measured at 532 nm. All experiments were performed in triplicates. The percentage of inhibition was calculated as explained previously.

Statistical Analysis

Experimental results are expressed as mean ± SEM. All measurements were replicated three times. The data were analyzed by a one-way analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

Qualitative phytochemical analysis

The preliminary qualitative phytochemical investigation documented that the peel extracts of all five citrus fruits showed the presence of many bioactive compounds *viz.* polyphenols, flavonoids, terpenoids, steroids, glycosides, alkaloids and carotenoids. The results also revealed that saponins were present in peel extracts of orange, citron and sour orange while absent in peel extracts of lime and pomello. The results of the analysis are shown in Table 1.

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Table 1: Qualitative phytochemical analysis of five Citrus fruits peel extracts

Tests	Lime	Orange	Sour Orange	Pomello	Citron
Steroids	+	+	+	+	+
Glycosides	+	+	+	+	+
Terpenoids	+	+	+	+	+
Saponins	-	+	+	-	+
Alkaloids	+	+	+	+	+
Carotenoids	+	+	+	+	+
Flavonoids	+	+	+	+	+
Tannins and Polyphenols	+	+	+	+	+

In Vitro Antiproliferative Activity by MTT Assay

The results of anti-proliferative activity of peels of citrus fruits at different concentrations (250, 500, 1000, 1500 and 2000 µg/mL) against MCF-7 and U-87MG were represented in Table 2 and in graphical representation Figure 1 and 2. The effect of the samples on the proliferation was expressed as the % cell viability. A significant decrease in percent of viability was observed in all extracts with increase in concentration. At 2000µg/mL concentration pomello, lime, orange, sour orange and citron revealed a percentage viability of 28.25±0.56, 29.76±0.77, 45.93±1.12, 43.49±0.57 and 47.56±0.48 respectively against MCF-7 whereas for U-87MG it was found to be 41.34±0.46, 26.43±0.21, 20.03±0.54, 22.56±0.34 and 28.31±0.26 respectively. Cisplatin was used as standard which exhibited a Percentage viability of 4.29±0.82 and 2.25±0.65 at 15 µg/mL concentration for MCF-7 and U-87MG respectively. Pomello peel extract showed considerable cytotoxicity and cell proliferation inhibition in MCF-7 cell line with an IC_{50} value of 872±0.43 µg/mL, followed by lime (978±0.55µg/mL), sour orange (1920±0.42µg/mL), orange (1965±0.83 µg/mL) and citron (2000±0.49 µg/mL) extracts. While for U-87MG cell line orange peel extract showed significant decrease in cell proliferation with an IC_{50} value of 778±0.768 µg/mL while sour orange, lime, citron and pomello exhibited 870±0.57, 1076±0.45, 1116±0.66and 1647±0.42 µg/mL IC_{50} respectively. From the results it was evident that different extracts exhibited different activity on cell lines. This selectivity could be due to the sensitivity of the cell line to the active compounds in the extract or to tissue specific response. However it was interesting to note that, there is a positive correlation between the antioxidant and anti-proliferative effects of the extracts.

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Table 2: *In vitro* Cytotoxic activity of Citrus fruits peel extracts on Glioblastoma cell line (U-87MG) and Breast cancer cell line (MCF-7)

Cell line	Concentration (µg/ml)	Inhibition (%)					Standard 15(µg/ml)
		Lime	Orange	Sour Orange	Pomello	Citron	
	Control	0	0	0	0	0	
	250	17.47±0.68	22.27±1.32	19.57±0.69	12.5±0.51	16.88±0.96	
U-87MG	**500**	27.27±0.35	38.98±0.82	36.64±0.93	23.23±0.49	26.92±0.52	97.74±0.65
	1000	49.75±0.93	59.54±0.88	56.58±0.08	36.31±0.47	47.59±0.54	
	1500	62.79±0.62	71.37±0.61	69.45±0.09	46.79±0.26	59.31±0.19	
	2000	73.57±0.21	79.97±0.54	77.44±0.34	58.66±0.46	71.69±0.26	
	250	16.8±0.29	12.68±0.63	6.76±0.58	19.82±0.14	5.13±0.79	
	500	31.63±0.51	20.05±0.67	13.02±0.74	36.55±0.68	11.12±0.51	
MCF-7	**1000**	53.31±0.54	29.1±0.67	24.78±0.45	56.75±0.4	23.25±0.67	95.70±0.82
	1500	61.92±0.16	37.75±0.75	35.22±0.52	62.77±0.35	33.66±0.88	
	2000	70.24±0.77	54.07±1.12	56.51±0.57	71.75±0.56	52.44±0.48	

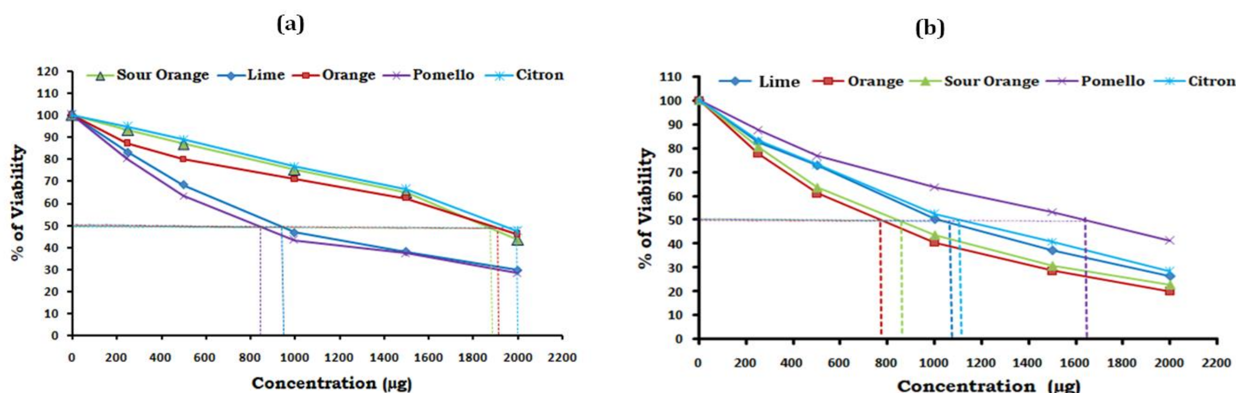

Figure 1: Graphical representation of antiproliferative effects of the Citrus Peel extracts on (a) MCF-7 cell line (b) U-87 MG cell line

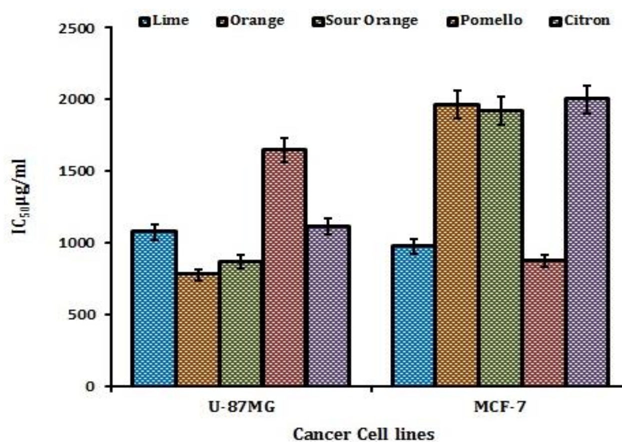

Figure 2: Graphical representation of IC_{50} of Citrus Peel extracts against U-87 MG and MCF-7

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Evaluation for *in vitro* antioxidant activities

ABTS radical scavenging activity

ABTS is a protonated radical that has a characteristic maximum of 734 nm, which decreases with the scavenging of proton radicals. The scavenging of ABTS radical increased with concentration. Fig. 3(A) shows the dose-dependent increase in ABTS radical scavenging ability of extracts and standard. The results were expressed in effective concentration (EC_{50}) which was found to be highest in pomello peel extract with an value of 262±1.08 µg/ml, followed by orange, lime, sour orange and citron with 295.25±0.5, 298.90±0.53, 331.12±0.6 and444.53±0.72 µg/ml respectively. The $EC_{50}$of standard ascorbic acid was 8.0±0.38 µg/ml.

Hydroxyl radical scavenging activity

Hydroxyl radical was generated by Fenton reaction, which degrades deoxy ribose. The results show a dose response increase in the capacity to quench hydroxyl radicals for all the concentrations studied (Fig. 3B). The scavenging activity of five peel extracts at varying concentrations was measured along with BHT. The EC_{50} values for fruit peel extracts were found to be highest in citron (652.75±0.35 µg/ml) followed by orange (697.86±0.35 µg/ml), lime (782.12±0.31µg/ml), sour orange (787.29±0.46 µg/ml) and pomello (909.32±0.55 µg/ml) respectively, whereas BHT has recorded an EC_{50} value of 40.06±0.32 µg/ml.

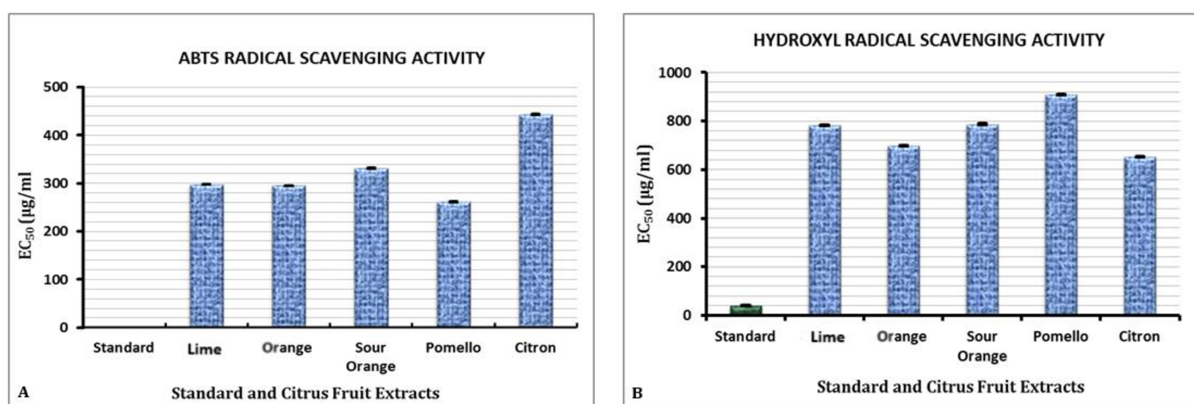

Figure 3: *Invitro* antioxidant activities (EC_{50}) of five Citrus fruits peel extracts

(A) ABTS radical scavenging activity (B) Hydroxyl radical scavenging activity

The use of plants as source of medicines for treatment of infectious and non-infectious diseases is an old human tradition [28], and the practice is now increasing due to increased global health challenges [29]. About 80% of people worldwide employ natural remedies to prevent or treat several illnesses. Since ancient times, edible plants and their fruits have been the basis of many traditional medicines, continuing to provide humankind with new remedies Furthermore, 50% of anticancer drugs used in clinical trials have been isolated from plants [4,30]. Consequently, traditional medicinal plants can serve as potential sources in the development of new, more effective anticancer agents for future therapy. The consumption of fruits and vegetables are an essential part of balanced

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diet and has been associated with low incidences and mortality rates of cancer [30]. Eventhough citrus fruits have been the target of major pharmacological studies, most of them are concentrated in the fruit pulp while their peels have not been subjected to such rigorous studies. In the present study, MTT assay was used to determine the *in vitro* antiproliferative activity or cytotoxicity of citrus peel extracts against U-87MG and MCF-7cells. Further their antioxidant potential was assessed by ABTS and Hydroxyl radical scavenging activity. Among the enzyme-based assays, the MTT assay is the best known method as it measures cell membrane integrity by determining mitochondrial dehydrogenase activities in the living cells on the reduction of MTT to a purple formazan by NADH [31]. From the results it was revealed that all the extracts under study possessed antiproliferative activity against both the cell lines however it is quite noticeable against U-87MG. Pomello extract exhibited significant antiproliferative effects on MCF-7 while orange peel extract showed superior activity against U-87 MG. Most of mutagenic and carcinogenic agents display their destructive effects through free radicals including reactive oxygen's species (ROS). So that antioxidants are able to reduce ROS. ROS have a role in etiology of diseases such as cancer, cardiocellular, neural problems and senescence [9,11].The results of radical scavenging activities revealed that ABTS radical scavenging assay was highest in Pomello whereas Hydroxyl radical scavenging activity was found to be more in citron peel extract. Therefore peel extracts studied manifested differential expression of cytotoxic and antioxidant capacity which may be due to their phytoconstituents operational under different mechanism. The current study also revealed that peels of five citrus fruits *viz.* lime, orange, sour orange, pomello and citron are endowed with the presence of several bioactive compounds *viz.* polyphenols, flavonoids, terpenoids, steroids, glycosides, alkaloids and carotenoids. Therefore the health promoting effects of citrus fruits often attributed to the presence of phytonutrients. Perusal of literature suggest that, the synergistic effects of diverse phytoconstituents present in citrus fruits are responsible for many biological properties, among which the well-known antioxidant activity and the modulation of intracellular key pathways involved in degenerative processes leading to chronic pathologies such as cancer. In particular, dietary flavonoids interfere with carcinogen activation, stimulate carcinogen detoxification, scavenge free radical species, control cell-cycle progression, induce apoptosis, inhibit cell proliferation, oncogene activity, angiogenesis and metastasis as well as inhibit hormones or growth-factor activity [32]. Moreover, the research has been carried out to find chemopreventive agents in Citrus fruit, Coumarins [33], Limonoids [34] and Flavonoids [35] more specifically Polymethoxy flavonones [3] had been reported to inhibit most prevailing cancer types. Furthermore, the study of structure activity relationship (SAR) of citrus flavonoids and cancer prevention was linked to the structural similarities between flavonoids and 17β-estradiol, suggesting interaction of flavonoids with estrogen receptors and also with estrogen metabolizing enzymes, such as cytochrome P450 enzymesCYP1A1 and CYP1B1, which are over-expressed in variety of tumor tissues are
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responsible for their anticancer property [36,37].

4. CONCLUSION

On the basis of the results obtained, it is concluded that peels of Citrus fruits in addition to their counterpart edible pulp possess significant radical scavenging and *invitro* antiproliferative activities against both cell lines under study. The broad range of activity of the extracts suggests that, multiple mechanisms mediated by the phytoconstituents are responsible for their potent cytotoxic and antioxidant activity. Further research is underway to purify and characterize the active principles and to evaluate these phytochemicals as nutraceuticals after the detailed examination of the effects of these agents on cell lines *in vivo.*

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