

## Exploring the Anticancer Activity of Grape Seed Extract on Skin Cancer Cell Lines A431

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### ABSTRACT

*In this study, grape seeds were extracted using ethyl acetate and petroleum ether by solvent-solvent extraction method. The phytochemical tests were performed to identify different phytochemical compounds present in the grape seed extract (GSE). Antibacterial activity of the GSE was determined using agar diffusion method against Gram-positive and Gram-negative bacteria. Gas chromatography-mass spectrometry (GC-MS) and Fourier transform infrared spectroscopy (FTIR) analysis was done to identify the presence of bioactive compounds and their functional groups. The GC-MS results revealed a total of four compounds, known to have potent activity against cancer cells, viz, squalene, the most potent compound found in ethyl acetate extract and diethyl phthalate, ethyl-9-cis-11-trans octadecadienoate and (R)-(-)-14-methyl-8-Hexadecyn-1-ol in petroleum ether extract. Cytotoxic activity of the GSE was observed against skin cancer cell lines A4321 using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) MTT assay. The IC<sub>50</sub> value of the GSE against A431 skin cancer cell line was 480 µg/mL. This is first such report against A4321 cell lines. The study gives the overall perception about importance of GSE in medicine and nutraceuticals purposes.*

**Key words:** GSE (Grape seed extract), phytochemical, FTIR, GCMS, skin cancer cell line, anticancer agents

### INTRODUCTION

There are increased occurrence of diseases in the world due to various reasons among which the main cause is due to oxidative stress that are a major fact of concern. Oxidative stress is initiated by free radicals, causing protein and DNA damage along with lipid peroxidation and these changes contribute to different type of cancer and inflammatory diseases due to bacterial infections. Grape seeds are a good source of polyphenols, which have a potent antioxidant activity (Nirmala and Narendhirakannan 2011). Thus, grape seed extract (GES) can be used to treat deadly diseases like cancer and various bacterial infections.

In India an alarming rate of increase of skin cancer has been seen in recent times. In a statistical survey, it has been shown that total skin cancer patients in India in 2004 were 8, 19, 354, which has increased to 9, 62, 832 and 9, 79, 786 in 2009 and 2010, respectively (Ali et al. 2011). The most important skin cancer causing reason in India and other countries is the alarming rate of increase of urbanization, which results increased pollution due to the vehicles, smoke emitted from different types of industries, etc. These cause the depletion of ozone layer that results in the entry of ultra violet rays of sun into the earth and cause different types of mutations in human beings, which leads to skin cancer. Unhealthy food habits

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of urbanized people in modern world are also another reason for cancer.

Grape seeds are present in the pomace of wine industries. Phytochemicals such as alkaloids, terpenes, fats oils found in the GSE are responsible for various antimicrobial activity of the extract. Terpenoids present in the GSE are the main antibacterial compounds, which includes a complex of 2, 3 oxidosqualene, which disrupts the bacterial membrane (Penduka et al. 2013).

The main aim of the study was to prepare GSE by solvent –solvent extraction method, identifying various phytochemical compounds present in the extract, examining the antibacterial activity of the extract by agar diffusion method using Gram negative and Gram positive bacteria, performing GC-MS and FTIR analysis of the compound.

## MATERIAL AND METHODS

### Sample

Ripened red grapes were purchased from local fruit market of Vellore, Tamil Nadu, India. The grapes were de-stemmed, washed with water, crushed and seeds were separated. They were washed and dried at 40°C in hot air oven and powdered by using blender (Liu and White 2012; Ranjitha et al. 2014). It was stored in a freezer for further use.

### Grape seed extraction

Ethyl acetate and water was used in 9:1 ratio to extract the compounds from the grape seeds by liquid-liquid extraction method by taking 388 g of grape seed powder in 500 mL of solution mixture of ethyl acetate and water in an Erlenmeyer flask, stirred and kept for 24 h with periodic stirring. Then the solution was filtered using Whatman filter paper and dried under hot air oven at 45°C. Petroleum ether was used to precipitate the compound. Both the filtrates were dried by using rotary vacuum evaporator at 55°C and samples were used for further analysis (Liu and White 2012). The maximum percentage of the product obtained by solvent extraction method was calculated after evaporating the solvent from the powdered grape seeds (Kantha 2012).

### Phytochemical analysis

Qualitative analysis for the GSE obtained from both ethyl acetate: water solvent and petroleum ether: water solvent was done. Alkaloid,

carbohydrates, fixed oils, fat detection, tannins, saponin, terpenoids, steroids, flavonoid and cardiac glycosides tests were performed following the standard methods (Evans 1989; Khandelwal 2005; Egwaikhide and Gimba 2007; Yisa 2009; Ganatra et al. 2012; Kantha 2012).

### FTIR and GC-MS Analysis

FTIR Spectroscopy analysis for GSE was done at a wavelength range of 400 - 4,000  $\text{cm}^{-1}$  to detect the functional group present in a molecule based on their vibration frequency between the bonds of atom (Rubilar et al. 2013). GC-MS analysis was done by using a Perkin Elmer GC Claurus 680 system (Naine et al. 2014). Unknown component spectrum was compared with known component spectrum, which was stored in NIST. The molecular formula, molecular weight and number of hits were used to identify the name of the component of GSE from the NIST spectra library.

### Antibacterial activity

Antibacterial assay was done by the agar well diffusion method. Log phase bacterial cultures  $10^8$  CFU/mL of Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Pseudomonas* sp.) were swabbed uniformly on Muller Hilton agar plates. Different concentrations of GSE (25, 50, 75 and 100  $\mu\text{L}$ ) were added to the well. Sterile distilled water was used as a negative control. The plates were incubated at 37°C for 24 h. Diameter of the zone was measured in mm (Manasa et al. 2014).

### Anticancer activity against skin cancer cell lines

The MTT assay was used to assess the viability of the cells. The skin cancer cell lines A431 cells ( $5 \times 10^3$ ) were seeded per well into 96-well plates containing 100  $\mu\text{L}$  DMEM medium with 10 % FBS incubated at 37°C. Cells were treated with different concentration of test compound (10-500  $\mu\text{g}/\text{mL}$ ) for 24 h. Doxorubicin (10-500  $\mu\text{g}/\text{mL}$ ) was used as internal positive control and 100  $\mu\text{L}$  of DMEM was used as negative control; wells without any cells were considered as blank. After incubating in humidified incubator with 5%  $\text{CO}_2$  at 37°C for 48 h, 20  $\mu\text{L}$  of 5 mg/mL MTT diluted in PBS were added to each well and incubated for 4 h. Then 100  $\mu\text{L}$  of 10% SDS in 0.01M HCl solution was added to each well to dissolve the formazan crystals formed. The plates were covered with aluminum foil and kept in an

incubator for 12 h for dissolution of the formed formazan crystals. Amount of formazan was determined measuring the absorbance at 560 nm using a micro plate reader (Mosmann 1983).

## RESULTS AND DISCUSSION

The bioactive compounds extracted from 388 g of GSE using ethyl acetate: water extract yielded the dry weight of 354.2 g and 281.28 for petroleum ether extract. The yield percentage of ethyl acetate: water extract was 91.28% and 72.4% from petroleum ether extraction. The phytochemical analysis of the GSE from ethyl acetate : water solvent and petroleum ether revealed the presence of alkaloids, carbohydrates, fixed oil and fat, tannins, saponin, terpenoids, cardiac glycosides, steroids and flavonoids in the crude ethyl acetate: water solvent extract, while the cardiac glycosides and steroids were absent in the petroleum ether extract (Table 1).

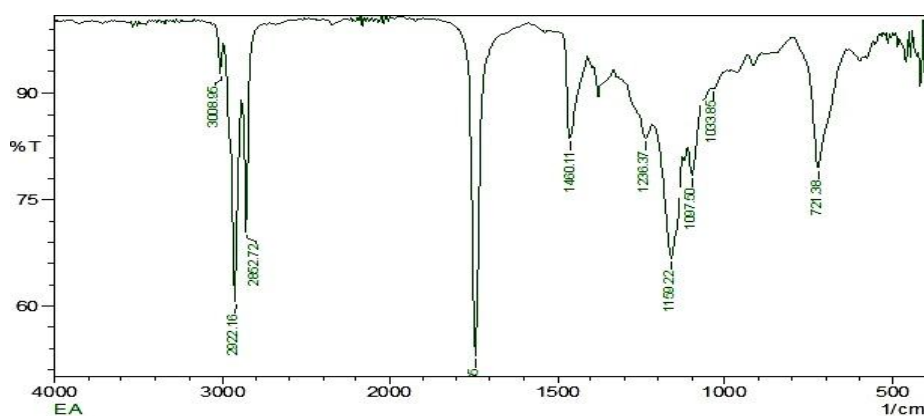
**Table 1** - Phytochemical Analysis of GSE.

Phytochemicals	Ethyl acetate Extract	Petroleum ether extract
Alkaloid test	+	+
Carbohydrate test	+	+
Fixed oil and fat test	+	+
Tannin test	+	+
Saponin test	+	+
Terpenoid test	+	+
Cardiac glycoside test	+	-
Steroid test	+	-
Flavonoid test	+	+

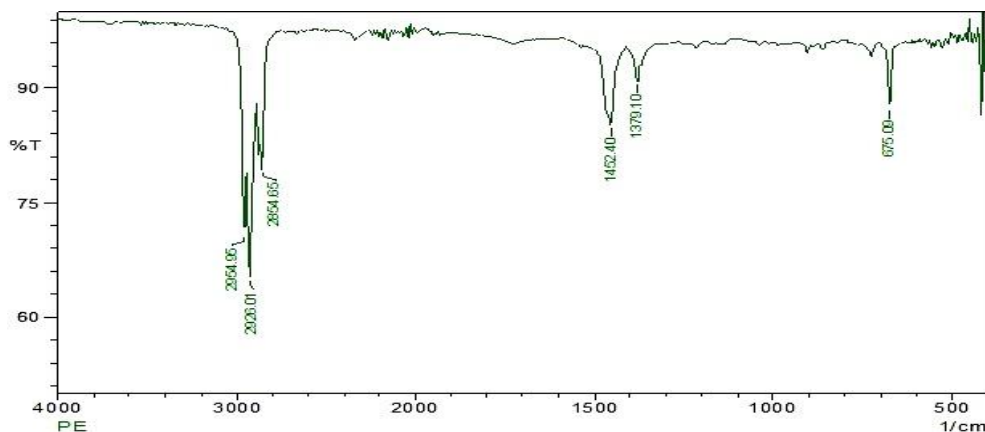
+ = positive, - = negative

## FTIR analysis

The FTIR analysis was done to identify the functional groups of the bioactive compounds present in GSE based on the vicinity of the infrared radiation. The peak absorbance results of FTIR analysis of GSE obtained from ethyl acetate: water and petroleum ether are shown in Figures 1 and 2, respectively. Figure 1 interpreted the data of infrared radiation (IR) spectrum of ethyl acetate: water extract, which showed strong absorption peaks at  $3008.95\text{ cm}^{-1}$  that indicated the C-H stretching vibration of the cis double bond ( $=\text{CH}$ ), asymmetric and symmetric stretching vibration of methylene ( $-\text{CH}_2$ ) bands were observed at peak  $2922.16\text{ cm}^{-1}$  and  $2852.72\text{ cm}^{-1}$ . Bending vibration of  $\text{CH}_3$  and  $\text{CH}_2$  aliphatic groups showed absorption at  $1460.11\text{ cm}^{-1}$ . Ester groups showed vibration of stretching mode from C-O at  $1236.37\text{ cm}^{-1}$  and  $1159.22\text{ cm}^{-1}$  and at peak  $1097.50\text{ cm}^{-1}$   $-\text{CH}$  bending vibrations of fatty acid was observed. Si-O stretching bend was absorbed at  $1033.85\text{ cm}^{-1}$  whereas overlapping Si-O stretching bend of methylene group ( $-\text{CH}_2$ ) was observed at peak absorbance of  $721.38\text{ cm}^{-1}$ . IR spectrum absorption of petroleum ether extract showed the asymmetric stretching vibration of methyl( $-\text{CH}_3$ ) group at peak value of  $2954.95\text{ cm}^{-1}$  and asymmetric and symmetric stretching vibration of methylene ( $-\text{CH}_2$ ) bands were observed at  $2926.16\text{ cm}^{-1}$  and  $2854.65\text{ cm}^{-1}$  (Fig. 2). Bending vibration of aliphatic groups  $\text{CH}_3$  and  $\text{CH}_2$  were seen at peak absorbance  $1452.40\text{ cm}^{-1}$ , whereas symmetric bending vibration of  $\text{CH}_3$  group was observed at  $1379.10\text{ cm}^{-1}$ . At peak value of  $675.09\text{ cm}^{-1}$ , bending vibration was seen in an alkene group ( $=\text{C-H}$ ).



**Figure 1** - FTIR Analysis of GSE extracted using ethyl-acetate: water solvent.



**Figure 2** - FTIR Analysis of GSE extracted using petroleum ether solvent.

### GC-MS analysis

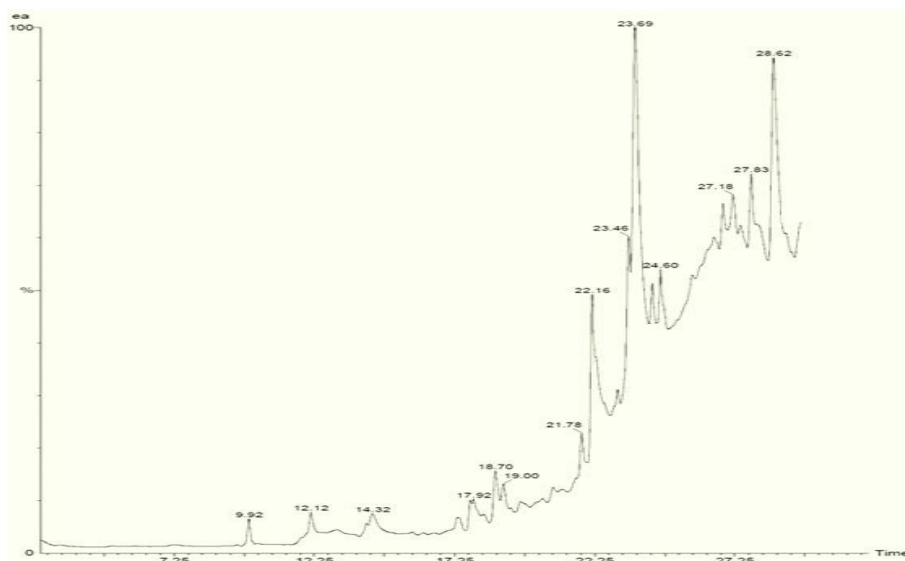
The GC-MS analysis identified few compounds present in the GSE having different therapeutic applications are shown in Figures 3 and 4. The molecular weight (MW), molecular formula (MF), retention time (RT), percentage of peak area and names of total 24 and 21 different compounds extracted from the ethyl-acetate and petroleum ether. Among 24 compounds extracted from ethyl-acetate, the most potential compound squalene belonged to titerpenoid family, which possessed antioxidant, antibacterial and anticancer activity (Bharathy and Uthayakumari 2013).

The structure of squalene (2,6,10,14,18,22 tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-e) is also a class of squalene, which shows

similar activities. Compounds belonging to organosilicon family, which are cyclohexasiloxane dodecamethyl (9.47%) and are active against fungi and bacteria (Ash and Ash 2004) such as 3-ethoxy-1,1,1,7,7,7-hexamethyl3,5,5-tris(trimethylsiloxy) tetrasilox (7.88%) used in deodorant, sun rays protection cream, in hair sprayer and also as cleaning agent (Valeri et al. 2013; Omoruyi et al. 2014), chloroacetic acid -10-undecenyl ester (7.71%) active against pest and also used in dye and in drug as a base (Koenig et al. 2005), and naphthalene, 5-ethyl-1, 2, 3, 4-tetrahydro (6.15%) belonging to monoterpenes family, active against the insects (Bogen et al. 2008).



**Figure 3** - Chromatogram of GSE extracted using ethyl-acetate: water solvent.

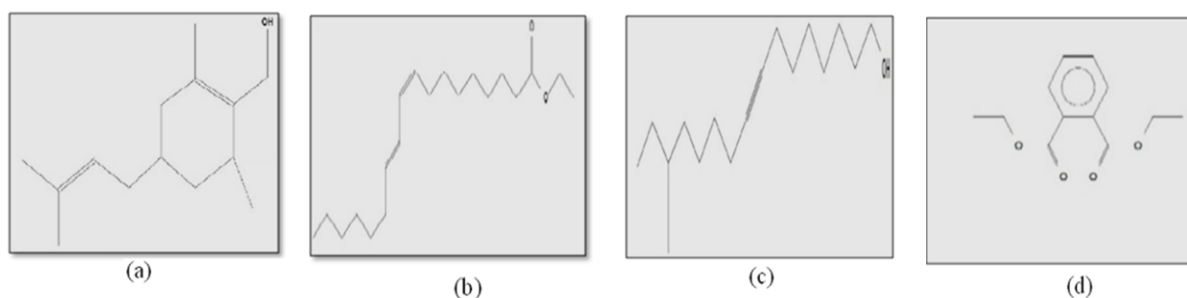


**Figure 4** - Chromatogram of GSE extracted using petroleum ether.

Compounds extracted from the petroleum ether had more applications in industry as well as in therapeutic medicine such as diethyl phthalate (9.55%), an ester of phthalate used as plasticizers in detergent as base and in aerosol spray (Ghorpade et al. 2002), di-(2-ethylhexyl) phthalate, a major bioactive metabolite proved with antimicrobial and cytotoxic property (Sayed 2012), and tetradecanoic acid, 10, 13-dimethyl-, methyl ester (9.37%), a saturated fatty acid used in detergent (Kale et al. 2011).

Compounds belonging to methyl ester of palmitic

acid, e.g., methyl 11-methyl-dodecanoate and (9.02%) used as flavouring agent (Lewis 1993) and heptacosanoic acid, 25-methyl-, methyl ester (8.99%) used for the antioxidant, anti-inflammatory and antimicrobial properties. Among the 20 compounds extracted by the petroleum ether, three were known to possess anti-cancer, which were ethyl-9-cis-11-trans octadecadienoate (8.90%), (R)-(-)-14,-methyl -8-hexadecyn-1-ol (8.35%) and diethyl phthalate(9.55%) (Habib and Karim 2012). The structures of these compounds are shown Figure 5.



**Figure 5** - Molecular structure and corresponding GC-MS peaks of major metabolites from GSE extract Squalene, (b) Ethyl- 9 -cis -11- trans octadecadienoate, (c) (R)-(-)-14,-methyl -8-Hexadecyn-1-ol (d) Diethyl phthalate.

#### Antibacterial activity test

The GSE were studied at different concentrations (50, 75 and 100  $\mu$ L) against two pathogenic bacterial strains, one Gram-positive (*S. aureus*) and one Gram-negative (*Pseudomonas* sp.). The petroleum ether GSE showed maximum activity

against *S. aureus* ( $17 \pm 0.09$  mm) and *Pseudomonas* sp. ( $20 \pm 0.09$  mm). The antibacterial activity of the extracts increased linearly with increase in the concentration of extracts (Table 2).

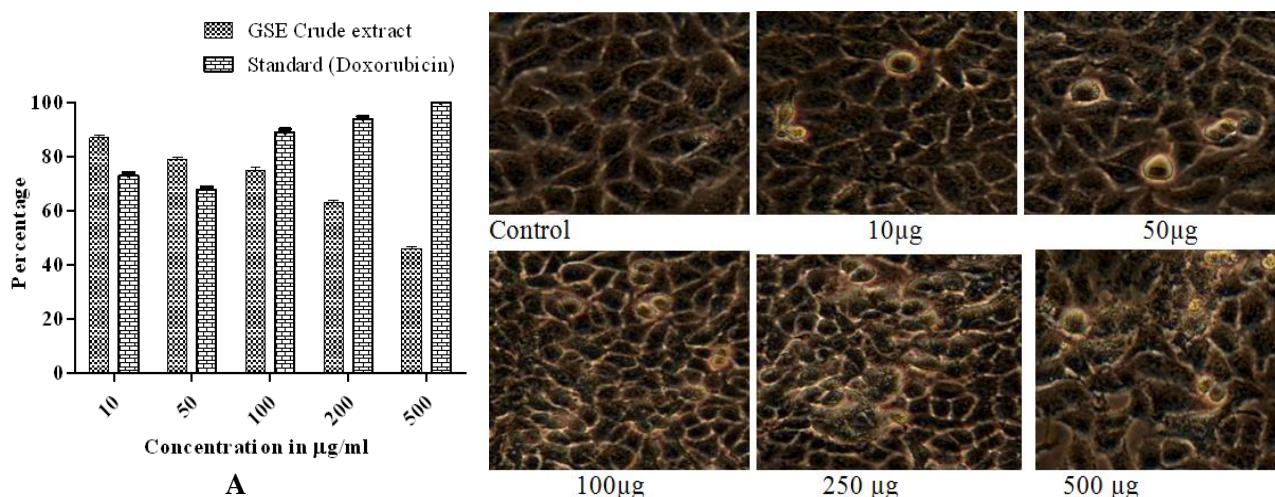
**Table 2** - Antibacterial activity of petroleum ether GSE.

Bacteria Strain	Zone of inhibition in mm			
	25 $\mu$ L	50 $\mu$ L	75 $\mu$ L	100 $\mu$ L
<i>Staphylococcus aureus</i>	-	10 $\pm$ 0.03	12 $\pm$ 0.05	17 $\pm$ 0.09
<i>Pseudomonas sp.</i>	-	15 $\pm$ 0.06	17 $\pm$ 0.04	20 $\pm$ 0.09

**Anticancer activity against skin cancer cell lines**

The petroleum ether extracted seeds analyzed for the cytotoxicity against skin cancer cell lines A431 showed maximum cell lysis at the concentration of 500  $\mu$ g/mL. The highest concentration showing maximum percentage of cancer cell lysis is shown in Figure 6A. The IC<sub>50</sub> value was 480  $\mu$ g/mL, which indicated that such concentration could lyse 50% of skin cancer cell lines. The morphological changes of treated A431 cell lines when visualized under a inverted light microscope showed detachment of the cells from the substratum, cell shrinkage, nuclear condensation and fragmentation (Fig. 6B). The morphological changes included reduction in the size of the cells and the cells

gradually became shrunken with the appearance of small bodies. The nature and the differential features occurred in the cancer cells clearly defined the cellular morphological characteristics, which was typical of cell death. Some studies have shown various pharmacological activities of the GSE with antioxidant, anticarcinogenic and anti-inflammatory properties (Li et al. 2008; Chen et al. 2012) This study gave the overall perception about importance of using GSE in medicine and nutraceuticals purposes with potential health benefits (Ky et al. 2015; Silva et al. 2015) The GSE could be used in ointments and also injected as injectable in skin cancer patients to treat skin cancer. Two major advantage of using GSE against skin cancer included it as a natural extract with no chemicals added (un-like the commercially available cancer drugs), hence there might be no chance of causing any side- effects to human body; the other advantage was its cheap cost.



**Figure 6** - (A) Percentage of cell viability represents the effective drug concentration toxic to A431 cell lines. (B) Morphological changes in A431 cell lines after treated with standard drug (Doxorubicin) and crude extract GSE.

**CONCLUSION**

The results revealed the fact that the GSE could be extracted using different solvents and the extract possessed many beneficial aspects for industrial and medicinal applications. The GC-MS analysis revealed that many compounds present in the grape seed extract could be used in industrial applications as a flavoring agents, cleansing agents, plasticizer, insecticides, pesticides,

antifungal, antioxidant and anti-inflammatory. The grape seeds showed several beneficial properties, which included it as a potent anticancer agent against skin cancer.

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## REFERENCES

- Ali I, Wani WA, Saleem K. Cancer scenario in India with future prospective. *Cancer Ther.* 2011; 8:56-70.
- Ash M, Ash I: Handbook of preservatives, Endicott, N Y: Synapse Information Resources 2004; 346.
- Bharathy V, Uthayakumari F. Bioactive Components in leaves of *Jatropha tanjorensis*. *JL Int J Pharm Res.* 2013; 59(4): 1839-1843.
- Bogen KT, Benson JM, Yost GS, Morris JB, Dahl AR, Clewell HJ, et al. Naphthalene metabolism in relation to target tissue anatomy, physiology, cytotoxicity and tumorigenic mechanism of action. *Reg Toxicol Pharmacol.* 2008; 51: 27-36.
- Chen BH, Hung MH, Chen JFF, Chang HW, Yu ML, Wan L, et al. Anti-allergic activity of grape seed extract (GSE) on RBL-2H3 mast cells. *Food Chem.* 2012; 132: 968-974
- Egwaikhid PA, Gimba CE. Analysis of the phytochemical content and anti-microbial activity of *Plectranthus glandulosus* whole plant. *Middle-East J Sci Res.* 2007; 2 (3-4): 135-138.
- Ghorpade N, Mehta V, Khare M, Sinkar P, Krishnan S, Rao CV. Toxicity study of diethyl phthalate on fresh water fish *Cirrhinamrigala*. *Ecotoxicol Environ Saf.* 2002; 53 (2): 255-258.
- Habib MR, Karim MR. Antitumor evaluation of di-(2-ethyl hexyl)phthalate (DEHP) isolated from *Calotropis gigantea* L.flower *Acta Pharm.* 2012; 62(4): 607-615.
- Kale SS, Darade V, Thakur HA. Analysis of fixed oil from *Sterculia foetida* linn. *Int J Pharm Sci Res.* 2011; 2(11): 2908-2914.
- Kantha DA. Wound healing and anti-genotoxic activities of *Aegle marmelos* with relation to its antioxidant properties. *J Pharm Res.* 2012; 5(3): 1492-1502.
- Khandelwal KR. A textbook of practical pharmacognosy. Nirali prakashan, 27<sup>th</sup> edition. 2012: 151-163.
- Koenig G, Lohmar E, Rupprich N. Chloroacetic acids, Ullmann's Encyclopedia of Industrial Chemistry, Weinheim: Wiley-VCH, doi:10.1002/14356007.a06\_537. 2005.
- Ky I and Teissedre PL. Characterisation of Mediterranean Grape Pomace Seed and Skin Extracts: Polyphenolic Content and Antioxidant Activity. *Mol.* 2015; 20(2): 2190-2207.
- Lewis RJ. Hawley's Condensed Chemical Dictionary", Van Nostrand Reinhold Co, 12<sup>th</sup> edition. 1993.
- Li H, Wang X, Li P, Lid Y, Wang H. Comparative study of antioxidant activity of Grape (*Vitis vinifera*) seed powder assessed by different methods. *J Food Drug Anal.* 2008; 16(6), 67-73.
- Liu SX and White E. Extraction and characterization of proanthocyanidins from grape Seeds. *The Open Food Sci J.* 2012; 6: 5-11.
- Manasa M, Vivek MN, Kambar Y, Kumar RKA, Kekuda PTR. Mineral content, antimicrobial and radical scavenging potential of *Caesalpinia mimosoides* Lamk (Caesalpinaceae). *World J Pharm Res.* 2014; 3(4): 1047-1063.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983; 65: 55-63.
- Naine SJ, Devi CS, Mohanasrinivasan V, Vaishnavi B. Bioactive potential of a strain *Streptomyces* sp.VITJS11 isolated from marine sediments. *Braz Arch Biol Technol.* 2014. (In Press)
- Nirmala JG, Narendhirakannan RT. *In vitro* antioxidant and antimicrobial activities of grapes (*vitis vinifera*) seed and skin extracts - muscat variety. *Int J Pharm Pharma Sci.* 2011; 3(4).
- Omoruyi BE, Afolayan AJ, Bradley G. The inhibitory effect of *Mesembryanthemum edule* (L.) bolus essential oil on some pathogenic fungal isolates. *BMC Complement Alter Med.* 2014; 14: 168.
- Penduka D, Buwa L, Mayekiso B, Basson AK, Okoh AI. Identification of the anti *Listerial* constituents in partially purified column chromatography fractions of *Garcinia kola* seeds and their interactions with standard antibiotics. *Hindawi Publishing Corp.* 2014; 8.
- Ranjitha CY, Priyanka S, Deepika R, Smitha Rani GP, Sahana J, Kekuda PTR. Antimicrobial activity of grape seed extract. *World J Pharm Pharm Sci.* 2014; 3(8): 1483-1488.
- Rubilar JF, Cruz RMS, Silva HD, Vicente AA, Khmelinskii I, Vieira MC. Physico-mechanical properties of chitosan films with carvacrol and grape seed extract. *J Food Eng.* 2013;115: 466-474.
- Sayed MH. Di-(2-ethylhexyl) Phthalate, a major bioactive metabolite with antimicrobial and cytotoxic activity isolated from the culture filtrate of newly isolated soil *Streptomyces* (*Streptomyces mirabilis* Strain NSQu-25) *World App Sci J.* 2012; 20 (9): 1202-1212
- Silva DF, Matumoto-Pintro PT, Bazinet L, Couillard C, Britten M. Effect of commercial grape extracts on the cheese-making properties of milk. *J Dairy Sci.* 2015; 98(3):1552-1562.
- Sunil H, Ganatra, Shweta P, Durge, Patil SU. Preliminary phytochemicals investigation and TLC analysis of *Ficus racemosa* Leaves. *J Chem Pharm Res.* 2012; 4(5): 2380-2384.
- Yisa J. Phytochemical analysis and antimicrobial activity of *Scoparia dulcis* and *Nymphaea lotus*. *Australian J Basic and Applied Sci.* 2009; 3(4): 3975-3979.

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