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ANTIOXIDANT, ANTIBACTERIAL EFFECTS OF SOLANINE ISOLATED FROM *SOLANUM NIGRUM* AND ITS CYTOTOXIC ACTIVITY ON THE HEP-2 AND AGS CELL LINES

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Keywords:

Solanine, Antioxidant,
Antibacterial, Cytotoxicity

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ABSTRACT: *Solanum nigrum* is a well known traditional medicinal plant. This study was intended to isolated active compound from the *Solanum nigrum* leaf. Solanine were isolated and purified from leaves of *Solanum nigrum* by using acid extraction along with alkaline precipitation methods and results shows that leaves of *Solanum nigrum* found to good sources of Steroidal glycol-alkaloids together with their structure were evaluated using various spectroscopic data of UV, FTIR, HPLC, NMR as well as MS. Antioxidative effects of solanine were tested in the *in vitro* (DPPH, superoxide anion radical and the hydroxyl radical) furthermore antibacterial activity were analysed against pathogens *E. coli*, *Staphylococcus aureus*, *Salmonella typhi* - A and *Pseudomonas aeruginosa* and its cytotoxicity effects were tested using Hep-2 and AGS cell line.

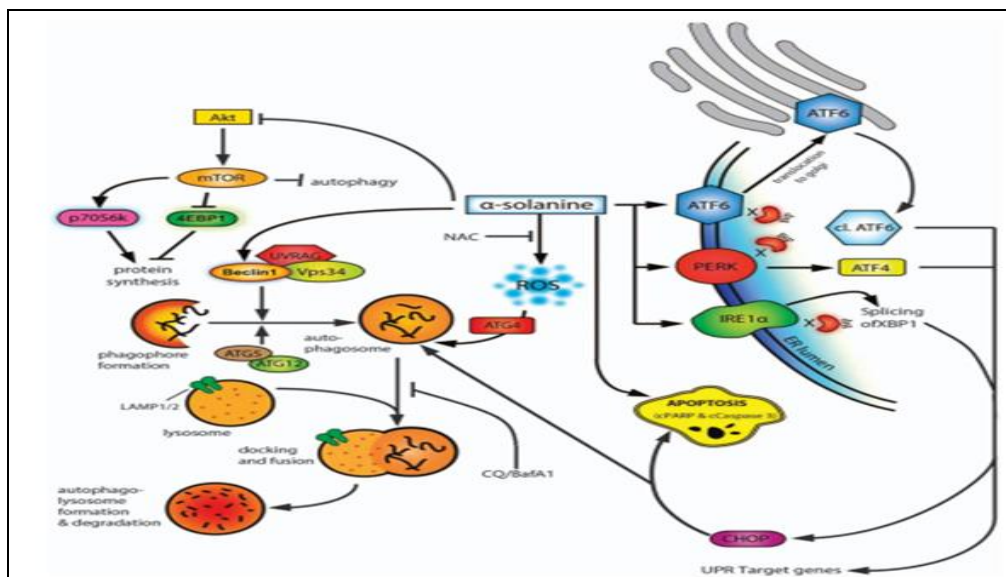
INTRODUCTION: Currently cancer researchers are focusing on developing therapeutic approaches with high specificity so as to improve response rates and to reduce side effects. Phytochemical therapy is one such strategy developed to improve the specificity and to reduce side effects to cancer treatment. Plant secondary metabolites and their semi-synthetic derivatives continue to play an important role in anticancer drug therapy. *Solanum nigrum* is a dicot plant under the family of Solanaceae, annual branched herb, the flowers are very small and five petals in the leaves, fruits are small. It's mainly found in waste lands and cultivated lands which are a common plant found in most parts of the world¹ and this plant as a reservoir of phytochemicals which have pharmacological activities².

Solanine was mainly present in the Solanaceae family mainly potato plant (*Solanum tuberosum* L.) and nightshade plant (*S. nigrum* L.)³⁻⁷. The green peel and the sprouts of the potato tuber contain high level of solanine expression⁸⁻¹⁰. The nightshade family of *Solanum nigrum* contains many steroid alkaloids such as Solamargine, solasonine, solanine and saponin etc., which used for antitumor therapeutics and shows the inhibitory effect on tumour growth in animal models and tumour cells of breast cancer¹¹⁻¹³.

Oral administration of *Solanum nigrum* derived polysaccharides against cervical cancer of tumour bearing mice. These contents increased of bax gene and decreased the Bcl - 2 and mutant p-53 expression. So these results demonstrated the apoptosis in tumour cells¹⁴. *Solanum nigrum* derived compounds of saponins and other alkaloids have antimicrobial and antioxidant activity reported¹⁵⁻¹⁶. *Solanum nigrum* derived glycoproteins have a dose dependent radical scavenging activity against many free radical of DPPH, OH, Superoxide anion and also induce apoptosis in HT-29 cells¹⁷⁻¹⁹.

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M Hasanain *et al.*, 2015 proposed the solanine induced apoptosis in cells.



Experimental Section:

MATERIALS:

Collection of Plant Materials: *S. nigrum* were collected from vadapalanji area, Madurai, Tamil Nadu, India. The collected plant materials were identified, authenticated by BSI and voucher specimens were deposited in the BSI Coimbatore. The dried leaves are used for the isolation of solanine. The *Solanum nigrum* of plant were shade dried, finely powered and subjected to isolation following the method of Siddiqui book of chemistry.

Isolation of Solanine: 100 gms of finely powered of *Solanum nigrum* leaf parts are mixed with 5% acetic acid (300 ml) in a beaker for 24 hours. Boiled of this material upto 2 hours and cooled. After cooling, pH of solution was adjusted upto 9.5 with ammonia or Sodium Hydroxide and again cooled. Centrifuged, washing the precipitate with sufficient amount of 1% ammonia solution and again centrifuged. Discarded the supernant liquid and washing. Residue was dried and weighed. In this way solanine was isolated. These isolated solanines were resuspended in respective solvents to get 100ug/ml for further analysis.

Confirmatory Test for Steroidal Glycoalkaloids:

To the chloroform layer bromothymol blue (BT) was added, shaken for 10 seconds and inferred with straw colored lower layer. Add sodium hydroxides which develop blue color, owing to the formation of aglycone - BT blue complex.

Confirmation Tests for Solanine:

Liebermann-Burchard Test: 2ml of acetic anhydride was added to the isolated solanine, each with 2ml of H₂SO₄. The colour changed from violet to blue or green in some samples indicate the presence of solanine.

Salkowask Reaction: Few mg of the residue of compound was taken in 2ml of Conc. Sulphuric acid was added from the side of the test tube. The test tube was shaken for few minutes. The development of red colour in the chloroform layer indicated the presence of Solanine.

Legals Test: A little fraction of compound was taken in solvent and adds alkaline solution was few drops of sodium nitro prusside solution. A blue color indicates the presence of glycosides.

Borntegers Test: A small fraction of compound was dissolved in 1ml of benzene and then 0.5ml of dilute solution of ammonia was added to the pink to red colour was indicative of the presence of glycosides.

Characterization of Solanine:

UV-Viible Spectroscopy: After chromatography, the TLC plate containing the solanine band were carefully removed by scrapping off the silica gel at the appropriate R_f value and eluted with chloroform. The eluted sample was analysed in UV-Visible spectrophotometer and compared with standard solanine. UV-visible spectroscopy

analysis was carried out on a SHIMADZU UV-visible absorption spectrophotometer.

FTIR Spectroscopy: The FTIR spectra of the solanine were recorded on Shimadzu FTIR 8000 series instrument. The FTIR spectrum was recorded within the frequency range between 4000-500 cm^{-1} and compared with standard solanine.

HPLC Analysis of Solanine: The solanine sample was further subjected to HPLC analysis. HPLC was performed (Shimadzu 9A model) using a reverse phase C18 column with a UV detector 20 μl of the sample were injected each time and detected at 232nm. The mobile phase was Methanol / Acetonitrile (60:40) at 1.0 ml min^{-1} the sample in the mobile phase was filtered through 0.2 μm PVDF filter before entering the column solanine was identified by comparing the peak area of the standard solanine (Sigma Aldrich).

ESI-MS Spectrum of Solanine: Mass spectra were recorded in LCQ Fleet mass spectrometer, Thermo Fisher Instruments Limited, US. Electrospray ionisation mass spectrometric method (ESI-MS).

NMR Spectrum of Solanine: The powdered Solanine was also subjected to $^1\text{H-NMR}$ (Bruker, 200 MHz in CDCl_3 ; internal standard TMS). The chemical shift values were recorded as δ (delta) value/ppm and the coupling constants are given in hertz (Hz).

To Find out the Total Antioxidant Activity of Isolated Active Compound: The isolated compound were tested for their scavenging activity against the stable free radical DPPH (2, 2'-diphenyl-1-picryl hydrazyl). The ability of isolated compound to scavenge DPPH was studied in a dot plot rapid screening assay and quantified using a spectrophotometric assay as proposed by Soler-Rivas *et al.*, (2000) and Mensor *et al.*, (2001) respectively.

Rapid Screening of Antioxidant Activity by Dot Plot Assay: Antioxidants react with diphenyl-1-picryl hydrazyl (DPPH) and convert it to diphenylpicryl hydrazine. The degree of discolouration from purple to yellow colour can be used as a measure of the scavenging potential of antioxidant extracts.

Aliquots (3 μl) of solanine were spotted on a TLC plate and allowed to dry. The TLC plate bearing the dry spots was placed upside down for 10 seconds in the solution of DPPH. The spots exhibiting radical scavenging, antioxidant activity showed up as yellow spots in a violet background. The intensity of the yellow colour depends on the amount and nature of the radical scavenger present in the spot.

DPPH Photometric Assay: The ability of the solanine to bleach DPPH can be quantified using a spectrophotometric assay, the extent of scavenging causing a proportionate change in the absorption at 518nm. An exact amount (0.5ml) of the methanolic solution of DPPH was added with extracts in the different solvents and the 0.48ml of methanol, and allowed to stand at room temperature for 30 minutes. Methanol served as the blank. After 30 minutes, the absorbance was measured at 518nm and converted into percentage radical scavenging activity as follows:

$$\text{Scavenging activity (\%)} = \frac{A_{518} [\text{Control}] - A_{518} [\text{Sample}]}{A_{518} [\text{Control}]} \times 100$$

Superoxide Radical Scavenging Assay: The superoxide radical generated from the photo reduction of riboflavin was detected by NBT reduction¹⁸. The reaction mixture contained EDTA (0.1 M), 0.0015% NaCN, riboflavin (0.12 mM), NBT (1.5 mM) and various concentrations of extract and phosphate buffer (67 mM, pH 7.8) in a total volume of 3 ml. The tubes were uniformly illuminated for 15 min and optical density was measured at 530 nm before and after the illumination. The percentage inhibition was calculated.

Hydroxyl Radical Scavenging Activity: Deoxyribose assay was used to determine the hydroxyl radical scavenging activity in an aqueous medium³⁴. The reaction mixture containing FeCl_3 (100 μM), EDTA (104 μM), H_2O_2 (1mM) and 2-deoxy- D-ribose (2.8 mM) were mixed with or without CPLL at various concentrations (10-250 μg) in 1ml final reaction volume made with potassium phosphate buffer (20 mM, pH 7.4) and incubated for 1 hr at 37 $^\circ\text{C}$. The mixture was heated at 95 $^\circ\text{C}$ in water bath for 15 min followed by the addition of 1ml each of TCA (2.8%) and TBA (0.5% TBA in 0.025M NaOH containing 0.02%

BHA). Finally the reaction mixture was cooled on ice and centrifuged at 5000 rpm for 15 min. Absorbance of supernatant was measured at 532 nm. Ascorbic acid was taken as the positive control.

Antimicrobial Activity: Gram Positive Bacteria – *Bacillus subtilis* and *Staphylococcus aureus*. Gram Negative Bacteria - *E. coli*, *Pseudomonas aerogenosa* one day prior to these testing, inoculations of above bacterial cultures were made in the nutrient agar. Preparation of medium (nutrient agar). It was prepared by dissolving definite volumes of nutrient agar in distilled water and sterilized by autoclaving at 15 p.s.i. for 20 minutes. Firstly prepared sterilized nutrient agar medium and poured into the petridish (25ml). After that near about (0.1ml) bacterial inoculum were added into the different petridishes respectively. Isolated solanines (100 mg) were dissolved in acetone to give a 100 g/ml of these stock solution was pipette out with help of micropipette and used for testing. After incubation, the zone of inhibition was measures in mm and it was reported. Tetracyclin was used as a standard drug.

Anticancer Activity in Cell Line:

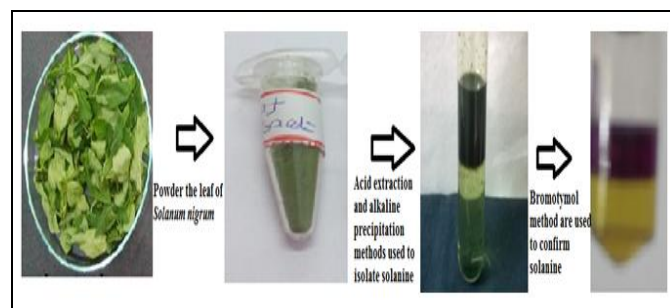
Cell Line: HEP-2, and AGS Cell lines was obtained from the National Centre for Cell Sciences, Pune, India, and were grown in DMEM media supplemented with 10% FBS 100 IU/ml, penicillin 100 mg/ml, streptomycin 20 mg/ml. The cells were maintained as monolayers in 25cm² plastic tissue culture flasks at 37 °C in a humidified atmosphere containing 5% CO₂ in air. Exponentially growing cells were used in all the experiments.

In-vitro Cell Viability Studies: The MTT assay is a simple, nonradioactive colorimetric assay to measure cell viability. Metabolically active cells are able to convert this dye into a water-insoluble dark purple formazan by reductive cleavage of the tetrazolium ring. Formazan crystals, then, can be dissolved in an organic solvent such as dimethylsulphoxide (DMSO) and quantified by measuring the absorbance of the solution at 545 nm, and the resultant value is related to the number of living cells. To determine cell cytotoxicity / viability, the cells were plated at a density of 1x10⁶ cells / well in a 96-well plate at 37 °C in 5% CO₂

incubator. After 24 hr of culture, the medium in the wells was replaced with the fresh medium containing solanine in varying concentrations. After 24 hr, 20 µl of MTT dye solution (5 mg/ml in phosphate buffer pH 7.4) was added to each well. After 4 hr of incubation at 37 °C and 5% CO₂, the medium was removed and formazan crystals were solubilized with 200 µl of DMSO and the solution was vigorously mixed to dissolve the reacted dye. The absorbance of each well was read on a microplate reader at 545nm. The spectrophotometer was calibrated to zero absorbance, using culture medium without cells. The relative cell viability (%) related to control wells containing cell culture medium without solanine compound was calculated by the following formula:

$$\% \text{ of cell viability} = 100 \times (\text{Sample absorbance} / \text{Control absorbance})$$

RESULTS: We isolate a solanine from *Solanum nigrum* plant using acid and alkaline precipitation methods its shown on **Fig. 1** after the isolation of solanine from *Solanum nigrum* we quantify the maximum concentration of solanine in mg/L when compare with commercially available solanine from sigma Aldrich. The isolated solanine was confirmed with many qualitative analysis as well as purity and structure prediction done by chromatographic methods (**Table 1**).



Confirmatory test for isolated solanine

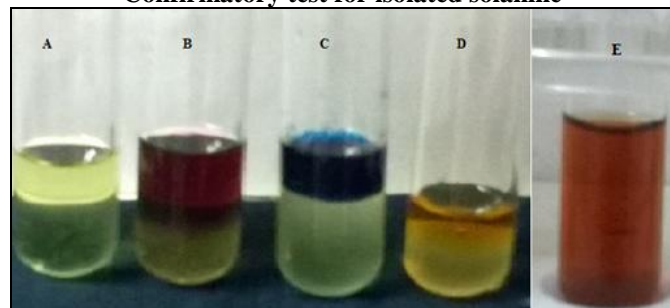
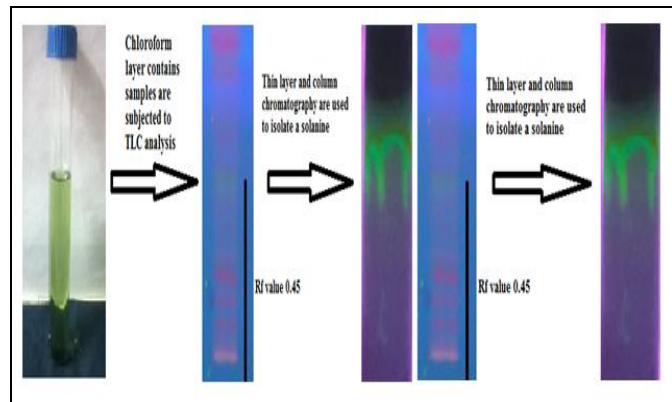


FIG. 1: ISOLATION AND COFIRMATION OF SOLANINE IN QUALITATIVELY AND QUANTITATIVELY USING BROMOTHYMOL METHOD

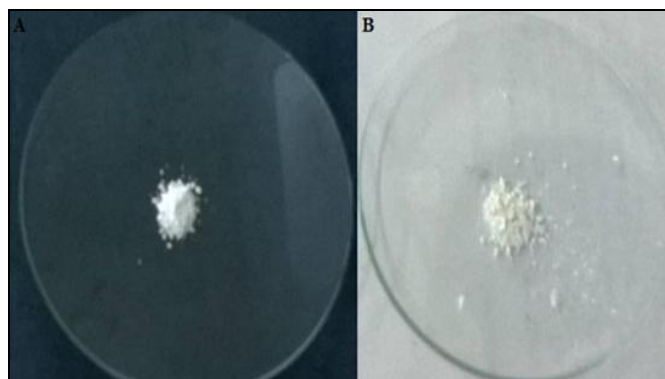
TABLE 1: CONFIRMATORY TEST FOR ISOLATED SOLANINE

S. No	Test	Colour changes observed	Presence or Absence (+/-)
1	Liebermann-Burchard test	Blue	+
2	Salkowask Reaction	Red	+
3	Legals test	Blue	+
4	Borntragers test	Pink	+

The isolated α -solanine was characterized and compared with the reported solanine. Initially the preliminary techniques such as thin layer chromatography and Melting points were tested for the isolated compound. The isolated compound was exactly matching with the commercially available solanine in thin layer chromatography **Fig. 2**.

**FIG. 2: THIN LAYER CHROMATOGRAPHY ANALYSIS OF ISOLATED SOLANINE**

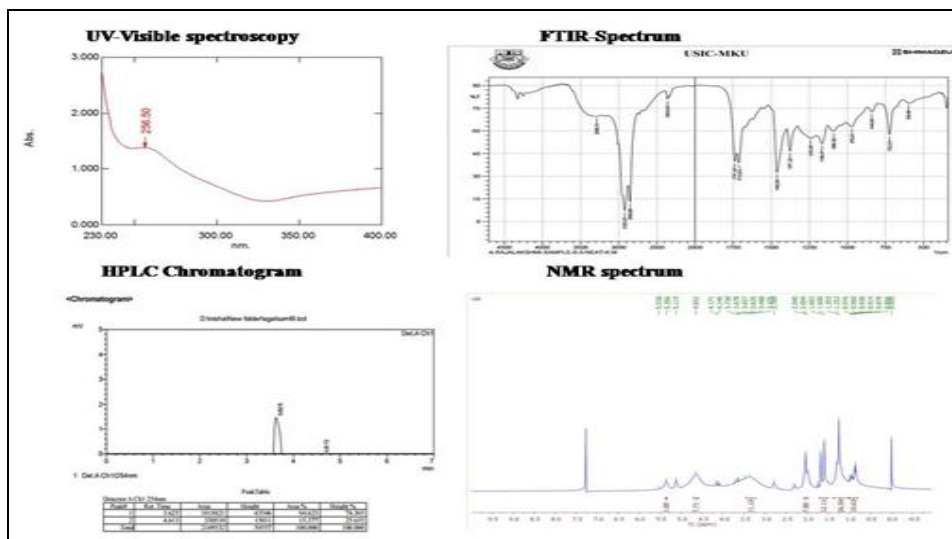
Similarly, the **Fig. 3** the melting point for the isolated solanine was tested, at the range of 268 – 270 °C.

**FIG. 3: MELTING POINT CHECKED FOR ISOLATED SOLANINE COMPOUND**

A. Reported Solanine (Melting point 271-273 °C)

B. Isolated Solanine (Melting point 268-270 °C)

The reported melting point is 271 - 273 °C confirms the presence of solanine. Further, characterization studies confirmed using the standard instrumental techniques such as Nuclear magnetic resonance spectroscopy, ESI-Mass spectroscopy and FT-IR spectroscopy. The FT-IR spectrum gives clear information for the presence of more hydroxyl group in the compound. Similarly the band appeared around 2900 cm^{-1} (C-H stretching frequency) indicates the presence of C-H units (**Fig. 4**). The compound contains several aliphatic units. The band around 1700 cm^{-1} indicates the presence C=C unit. Correspondingly the band around 1000 cm^{-1} indicates the presence of C-O bond stretching frequency. On continuation the isolated compound was confirmed by ESI-MASS spectroscopy. In the mass spectrum **Fig. 5** the positive mode peak appeared as 868.46 (M^+), which compared to actual molecular weight of solanine 867.

**FIG. 4: CHARACTERIZATION OF ISOLATED SOLANINE**

Finally, the compound was confirmed by NMR spectroscopy. The ^1H NMR clearly indicates the isolated compound contains approximately 74 protons. The proton count of solanine is 74. The peak around 5.3 ppm of one proton indicates the presence of olefinic proton. Similarly the peak around 3.0 to 5.0 ppm indicates the presence of hydroxyl unit and O-CH units. All the peaks were appeared in aliphatic region (0.5 – 5.0 ppm). The solanine does not contain any aromatic units most of them hydrocarbon unit, which will be appeared at aliphatic region. In addition the compound was confirmed by ^{13}C NMR, which is also explain the presence of aliphatic carbon unit. From the above all results we have evidently confirmed the isolated compound is α -solanine (Fig. 4).

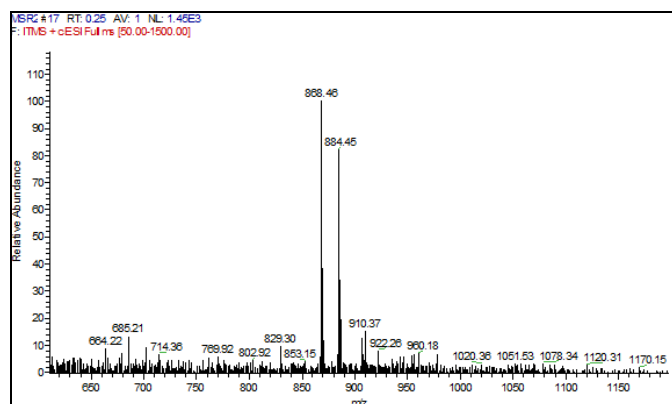


FIG. 5: ESI MASS SPECTRUM OF SOLANINE

The isolated solanines have a potential scavenging activity. The antioxidant assays are based on standard method of free radicals (DPPH), we performed the qualitative and quantitative analysis of antioxidant activity. In qualitative analysis, we followed by the dot Plot assay (Fig. 6).

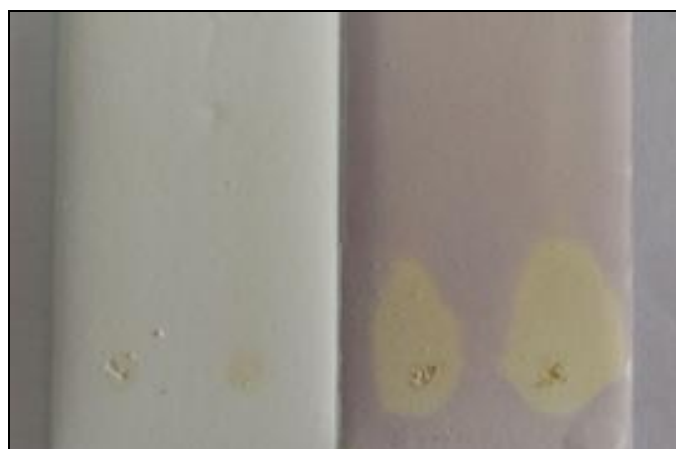


FIG. 6: DOT PLOT ASSAY

The violet background change into yellow indicated the scavenging effects of solanine

The free radical of DPPH spotted on TLC plate and then solanine compound were added in TLC plate the yellow spot were formed in violet background this reaction was indicates the scavenging activity of solanine (Figure shows). Evaluate the scavenging activity followed by DPPH Photometric assay. The DPPH dissolved in methanol and the ascorbic acid is used as a standard. 0.5ml of DPPH solanine dissolved in 0.5ml of DPPH solution and examine in UV spectrophotometer at the wavelength of 518nm. The Fig. 7 showed the solanine has higher scavenging activity (82%). For further analysis formation (or) neutralize the free radicals in our system used many techniques (SO, NO) Results are shown in the Table 2 and 3.

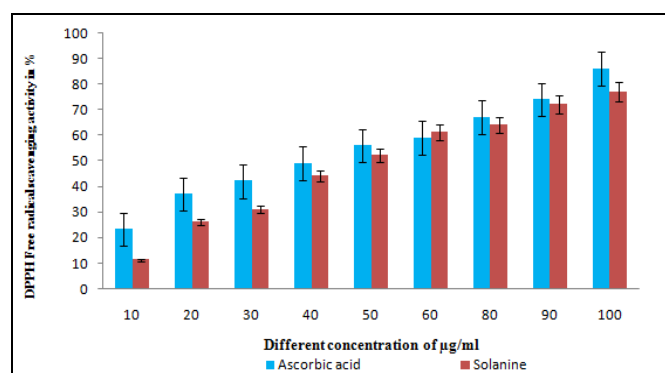


FIG. 7: DPPH PHOTOMETRIC ASSAY

TABLE 2: SUPEROXIDE RADICAL SCAVENGING ASSAY

Solanine (µg/mL)	Inhibitory effect of super oxide anion radical (%)
5	35.72
10	47.55
50	63.84
100	70.90

TABLE 3: HYDROXYL RADICAL SCAVENGING ACTIVITY

Solanine (µg/mL)	Inhibitory effect of Hydroxyl radical (%)
5	34.72
10	38.79
50	42.07
100	60.22

The result of this study indicates the good free radical scavenging ability is there in the isolated compound. Antibacterial and cytotoxic effects to the cells. But the cells are showing the drug treatment of solanine induces the apoptosis changes in the morphological behaviours (Fig. 8 and Fig. 9).

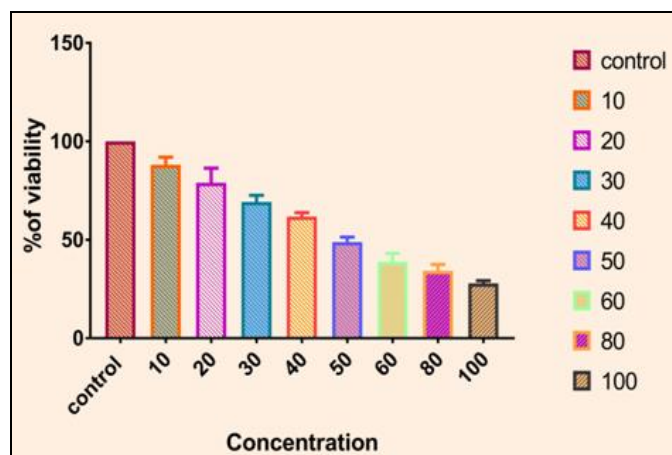


FIG. 8: MTT ASSAY IN HEP-2 CELL LINE

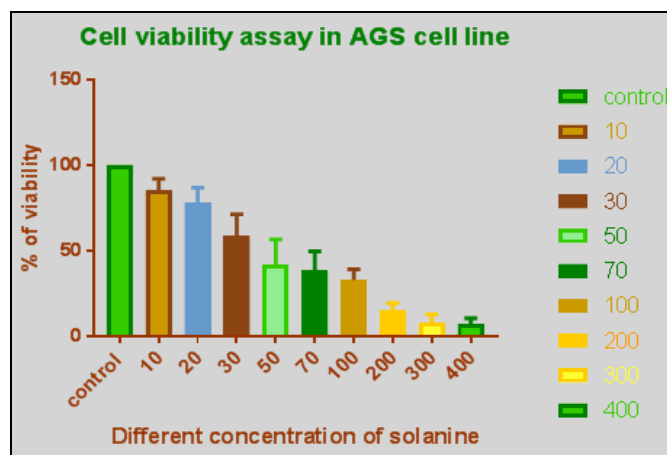


FIG. 9: MTT ASSAY IN AGS CELL LINE

TABLE 4: ANTIBACTERIAL ACTIVITY OF SOLANINE

S. No	Microorganism	Zone of inhibition in mm				
		Chloroform	Antibiotic control (Tetracycline)	20µg	10µg	5µg
A	<i>Bacillus subtilis</i>	-	11	-	-	-
B	<i>Staphylococcus aureus</i>	-	10.5	4	3	2.5
C	<i>Pseudomonas aeruginosa</i>	-	9	3	3.5	2.8
D	<i>E.coli</i>	-	10	8	4.2	1.7

DISCUSSION: Based on reported evidence the *S. nigrum* contain steroidal glycoalkaloids like solamargine, solanine, solasonine, diosgenin, steroidal sapogenins and diosgenone which has been reported²¹ which induce apoptosis in HepG2 Cells. Further report of²² the steroidal glycol-alkaloid of solamargine to exhibit the anti-tumor effects suggested the solamrgine and solasodine from *S. nigrum* induced a cytotoxic to Hep3B cells²³. Our result similar to this the isolated solanine molecules induced a toxicity to Hep-2 and AGS cell and decreased the viability at the concentration of 50 µg and 30 µg/ml. α-solanine have potent antibacterial activity against pathogenic bacteria depending on the concentration of the solanine showed the inhibition^{24, 25} so our results also investigated the 2 pathogenic microorganisms treated with solanine have inhibition against *Bacillus subtilis* and *Staphylococcus aurues*.

Challenges and Future Directions: Solanine is an alkaloid toxin found in members of the nightshade family, such as eggplants, potatoes, tamatoes, and the infamous deadly nightshade or belladonna. This toxin is part of the plant's defense mechanism, and it is designed to make nightshades unappealing and deadly to animals which might attempt to eat them.

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