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Studies on the Investigation of Anti-Oxidant and Cellular Toxicity on L929 Cell Line of *Andrographis paniculata* and its Derivative of Silver and Gold Nanoparticles

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ABSTRACT: The current pharmaceutical industry is increasingly turning to plant-based multi-potential bioactive compounds to combat a variety of diseases and pathological conditions due to the consequences of administrating synthetic drugs. To Avoid back reflection of synthetic drugs, researchers are focusing on natural derivatives guided from traditional medical practices. Plants are playing vital role in nutritional and disease control aspects in traditional era. Terrestrial resources contain a vast array of medicinally valuable plants that have traditionally been used to treat a wide range of diseases, including hepatoprotective, anti-inflammatory, antibacterial, anti-cancer, and antidiabetic properties. Similarly, the study plant *Andrographis paniculata* demonstrated significant medicinal properties; thus, the potential activity of a leaf ethanolic extract of *A. paniculata*, diethyl phthalate purified from the crude ethanolic extract, and their modified metal (Silver and Gold) nanoparticles against free radicals, namely DPPH and phosphomolebdenum assays. In addition, the study samples were subjected to an MTT assay to assess cellular toxicity in comparison to the L929 cell line. The results showed that the promising drug carrier system of diethyl phthalate silver nanoparticles (DPAgNPs) exhibited lower toxicity and higher anti-oxidant activity.

Keywords: *Andrographis paniculata,* Diethyl phthalate, Silver and gold nanoparticles, DPPH, Phospohomolebdenum and Cytotoxicity.

INTRODUCTION

Countless plant-based products, such as herbal teas, nutritional supplements, health meals, and other goods, are readily available today (Phillips and Meilleur 1998). Distinct medical traditions are practised by various cultural and ethnic groups (Leslie and Young 1992). In underdeveloped nations, where 65 to 80 percent of the world's population resides, plant products are their primary source of healthcare (Farnsworth *et al*., 1985; Awoyemi *et al*., 2012). Ayurveda has been demonstrated to be one of the oldest medical systems that is still in use today, both in India and around the world. Many individuals think natural treatments are safer than synthetic medications. Individualized therapies may incorporate nutritional, physical activity, and lifestyle factors as well as proprietary molecules or plant components (Sharma and Mujundar 2003). There has been a remarkable return of interest in medicine and classical pharmacopoeia despite substantial advancements in contemporary medicine. Perumal and Gopalakrishnakone (2008) explored plant-based medications, which significantly improved on already available therapies. Plants continue to play a key role in the control of diabetes, particularly in much less developed countries where the bulk of the population has little resources and no access to contemporary therapy. Alternative diabetes therapies, such as those based on plants, are becoming more and more popular in industrialised nations due to the drawbacks of taking insulin and oral hypoglycemic medications (Marles and Farnsworth 1994). In India, where more than 30 million individuals have the condition, diabetes is on the increase. Numerous members of society are impacted by undiagnosed diabetes. Both diabetes screening and the streamlining of diagnostic processes have become crucial. The two most prevalent kinds of diabetes are Type-1 and Type-2 (Gupta *et al*., 2014; Wang *et al.,* 2001). Phenolic compounds are a significant category of the plant secondary metabolites that have been found to have antioxidant properties. There are several findings on how phenolic chemicals affect the antioxidant capacity of various plant species (Cai *et al*., 2004). Diverse pharmacological effects of *Andrographis paniculata* exist, some of which include anti-cancer, anti-diarrheal, and anti-hepatitis (Md. Sanower Hossain *et al*. (2014). *A. paniculata* extracts show hepatoprotective qualities, antihypertensive

effects, and lower plasma angiotensin converting enzyme (ACE) activity and renal lipid peroxidation (Akbar, 2011). Besides, they have antimicrobial, anti-inflammatory, antioxidant, and hypoglycemic effects (Zhang and Tan 2000). In an effort to discover a treatment for conditions like rheumatoid arthritis and ulcerative colitis that are brought on by oxidative stress and infections, Umadevi and Kamalam (2014) at the University of Bristol investigated *A. paniculata*. They are looking at the phytochemical composition and antioxidant capacities of the plant. Similar to this, 10% methanolic SLE showed enhanced antioxidant activity, which was responsible for 53.94 g/ml DPPH activity and 44.94% suppression of nitric oxide activity (Uthirapandi *et al*., (2021). Penicillin has a wide range of possible uses, including as an anti-microbial, cytotoxic, anti-protozoan, and immunostimulant, according to a review by Okhuarobo *et al*. (2014). Andrographolide, the primary active ingredient, has a variety of biological effects, including hepatoprotective, anti-inflammatory, antibacterial, anti-cancer, and antidiabetic ones (Jarukamjorn and Nemoto 2008). Researchers suggest that andrographolide be structurally modified in order to acquire diverse leads due to the outstanding diversity of these biological functions. Numerous andrographolide compounds have surfaced in recent decades, and their pharmacological properties have also been examined. There haven't been many researches that thoroughly summaries or analyze *A. paniculata* and its derivatives, nevertheless. As a result, this study gives comprehensive information about the pharmacological activity of *A. paniculata* and its main ingredient andrographolide in an effort to advance the trends of research on andrographolide (Jayakumar *et al*., 2013). Antioxidant defences are strengthened by andrographolide. By scavenging free radicals, it exerts direct action. It further causes indirect interference by preserving mitochondrial integrity, preventing pro-oxidant enzyme activity, and/or activating antioxidant enzymes. Keep in mind that the control of the antioxidant defense system involves the transcription factor Nrf2. As a result, andrographolide's regulation of Nrf2 is important for controlling the redox system (Li *et al*., 2018; Tan *et al*., 2018; Yan *et al*., 2018).

MATERIAL AND METHODS

Sample Preparation. Around 50 g of powdered *Andrographis paniculata* was taken into fresh extract cloth and bagged into extraction apparatus. The system was placed on the heating mantle at 40°C and the sample with reflux condenser tube was placed over the solvent collector with air tightly. The extraction system was started with 500 ml ethanol as extraction solvent for 24 hours. After extraction the solvent was transferred to fresh 500 ml conical flask, filter the extract and stored at 4 °C for further analysis. Diethyl phthalate was partially purified from the crude extract of *A. paniculata* using column chromatography and has been characterised spectrally. From the diethyl phthalate chromatographic fraction, silver and gold nanoparticles were synthesised using ascorbic acid as a reducing agent and subjected to spectral and morphological characterization.

Anti-Oxidant Property

DPPH radical scavenging activity. The free radical scavenging activity of the fractions was measured in vitro by the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay according to the standard method (Brand-Williams *et al.,* 1995). The stock solution was prepared by dissolving 24 mg of DPPH in 100 ml of methanol stored at 4–20 °C. About 4 ml of various concentrations of samples (20, 40, 60, 80, and 100 g/mL) were mixed with 1 ml of methanolic solution containing DPPH radicals, resulting in a final concentration of DPPH of 0.2 mM. The mixture was shaken vigorously and left to stand for 30 minutes, and the absorbance was measured at 517 nm. Ascorbic acid was used as a control. The percentage of DPPH decolorization in the sample was calculated according to the equation. The control was prepared without any sample, and scavenging activity was estimated based on the percentage of DPPH radicals scavenged using the following equation:

% of inhibition = [(control OD–sample OD)/(control OD)]×100

Total antioxidant activity. Each test tube contained 3 mL of distilled water and 1 mL of Molybdate reagent solution, as well as 20-100 µg/mL of *Andrographis paniculata* extract, Diethyl phthalate (DP), DP silver nanoparticles (DPAgNPs), and DP gold nanoparticles (DPAuNPs). These tubes were kept incubating at 95 ºC for 90 min. After incubation, these tubes were normalized to room temperature for 20–30 min, and the absorbance of the reaction mixture was measured at 695 nm. The percentage of inhibition values from samples was calculated for each extract. Ascorbic acid was used as a positive reference standard. The PM assay is based on the reduction of phosphate-Mo (VI) to phosphate-Mo (V) by the sample and the subsequent formation of a bluish-green phosphate/Mo (V) complex at an acidic pH. The phosphomolybdenum method is routinely applied in the laboratory to evaluate the total antioxidant capacity of plant extracts (Prieto *et al*., 1999).

Cellular Cytotoxicity on L929 Cell line by MTT assay. This assay was performed based on the assessment of reduction of the yellow colored water soluble tetrazolium dye MTT to formazan crystals (MTT Cell Proliferation Assay Instruction Guide). Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals formed in assay mixture. Seed 200µl cell suspension in a 96-well plate at required cell density (20,000 cells per well), without the test agent. Allow the cells to grow for about 24 hours. Add appropriate concentrations of the test samples (Diethyl phthalate, DPAgNPs and DPAuNPs) incubate the plate for 24hrs at 37°C in a 5% CO_2 atmosphere. After the incubation period, takeout the plates from incubator and remove spent media and add MTT reagent to a final concentration of 0.5mg/mL of total volume. Wrap the plate with aluminium foil to avoid exposure to light. Return the plates to the incubator and incubate for 3 hours. (Note: Incubation time varies for different cell lines. Within one

experiment, incubation time should be kept constant while making comparisons). Remove the MTT reagent and then add 100µl of solubilisation solution (DMSO). Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals especially in dense cultures. Read the absorbance on a spectrophotometer or an ELISA reader at 570 nm wavelength. % Cell viability is calculated using below formula:

% cell viability=[Mean abs of treated cells/Mean abs of Untreated cells] × 100

Statistical Analysis. The obtained data were interpreted and calculated as Mean, Standard deviation and ANOVA using IBM SPSS Statistical Software and represented the data in tables as well as graphs.

RESULT AND DISCUSSION

Phytochemical Screening

Anti-Oxidant Activity

DPPH Scavenging Assay. The study samples (*A. paniculata*, Diethyl phthalate, DPAgNPs and DPAgNps) were subjected to investigate the scavenging activity against 2-diphenyl-1-picrylhydrazyl (DPPH) and the scavenging effects were analyzed and calculated statistically. Among the sample Diethyl phthalate silver nanoparticles (DPAgNPs) were described in Table and Figure: 1 as higher scavenging activity 60.36 % at 100 µg/mL concentration while *A.paniculata* showed 44.94 % at 100 µg/mL. However, Diethyl phthalate and DP gold nanoparticles exhibited quite lower scavenging effect as 16.42 % and 20.83 % at 100 µg/mL concentration. The one-tailed ANOVA was performed and showed insignificance of activity among the sample at p-value equals 1.738 (1.738 > p 0.005) greater than α value of 0.005 (Table and Fig. 2).

Total Anti-oxidant Activity. Total anti-oxidant property can be determined by analyzing the reducing activity of phosphate-Mo (VI) to phosphate Mo (V) was measured at 695 nm. In table and Figure: 3 Higher reducing property was shown in Diethyl phthalate silver nanoparticle (DPAgNPs) as 83.64 % at the concentration of 100 µg/mL while, DP gold nanoparticle exhibited lesser activity 14.29 % at 100 µg/mL. In other hand, Diethyl phthalate revealed 57.56 % and *A.paniculata* has 34.78 % of reducing activity at 100 µg/mL. The data of total anti-oxidant property was far away to significant at 0.005 level (1.088 > p 0.005) showed in Table and Fig. 4.

Assessment of Cellular Cytotoxicity by MTT Assay. MTT assay is a colorimetric assessment based on the reduction of the yellow colored water soluble tetrazolium dye MTT to formazan crystals by mitochondrial lactate dehydrogenase produced by live cells which in turn represented the percentage of cellular cytotoxicity of L929 cell line. Among the sample group, DP gold nanoparticle showed 87.9 % cytotoxicity to the cell line at the concentration of 100 µg/mL. While, diethyl phthalate and DPAgNPs exhibited 90.13 and 89.68 % of toxicity at 100 µg/mL in Table 5 and Fig. 7 and 8. This revealed the content of concentration depending cellular toxicity against L929 cell line. The toxicity of study samples were analyzed through one-tailed ANOVA and the value showed the significance at p 0.005 and there is no significant difference between the means of any pair (Table 6 and Fig. 5 and 6).

DISCUSSION

The activities of catalase, superoxide dismutase, and glutathione S transferase are significantly increased when the aqueous extract of *A. paniculata* is administered orally at a variety of dosages. It highlights the antioxidant properties of the aqueous extract of AP, which may contribute to the anticarcinogenic effect by lowering oxidative stress (Verma and Vinayak 2008). According to a study, the antioxidant activity of an *Andrographis paniculata* aqueous extract was higher than that of an ethanol extract. The aqueous extract has a radical scavenging activity of 66.8% at 50 µg/mL compared to 57.8% in the ethanol extract (Mussard *et al*., 2019). Methanolic plant extracts were most effective in scavenging DPPH free radicals and H_2O_2 radicals, with 50% inhibition at concentrations of 333.34 µg/ml and 398.12 µg/ml, respectively (Sinha and Raghuwanshi 2020). The aqueous stem extract (4.42 µg/mL) and ethanolic stem extract (6.84 µg/mL) had the lowest IC50 values, respectively. As a result, the aqueous extract of stem is the most effective in neutralising free radicals produced by the oxidation of lipids, proteins, and nucleic acids (Polash *et al*., 2017). The results of the present research conducted by Khan *et al.* (2020) showed that andrographolide decreased DLD1 cells ability to divide in a concentration- and time-dependent way. Through nuclear condensation, phosphatidylserine externalisation, and caspase-3 activation, andrographolide triggered apoptosis. Additionally, it increased the levels of cellular ROS, which were linked to the activation of apoptosis in DLD1 cells. Additionally, andrographolide showed synergistic efficacy against DLD1 cells when combined with 5-FU and PTX. According to methodology, plant component, and type of dietary supplement, antioxidant activity ranged from 503.36 to 6164.09 µmol TE/100 g d.m. when tested using the FRAP, CUPRAC, and DPPH procedures (Marzanna Kurzawa *et al.,* 2015). According to research on the anti-oxidant properties of *A. paniculata* ethanolic leaf extract, diethyl phthalate, DPAgNPs, and DPAuNPs against DPPH radicals and phosphomolebdenum, DPAgNPs has shown remarkable anti-oxidant activity at 100 µg/mL concentration, and the IC 50 value was 8.93 µg/mL for DPPH scavenging and 35.13 µg/mL for reducing phosphomolebdenum. Diethyl phthalate is an oily, colourless liquid with no odour or flavour. It can be found in toothbrushes, car components, toys, tools, cosmetics, aspirin, plastics, pesticides, and food packaging (World Health Organization, 2003). These findings imply that DEP exposure may cause sperm effects, hepatic effects, developmental effects, and androgen-independent male reproductive toxicity, with some indications of female reproductive toxicity. To thoroughly analyse these results and boost trust in this database, more study is required (Weaver *et al*., 2020). The growth of human

HaCaT keratinocytes in cell culture was significantly inhibited by andrographolide at 31.25 g/mL (90 M). To the best of our knowledge, this is the first time andrographolide from *A. paniculata* has been documented to have anti-proliferative effects. According to OECD test guideline No. 420, *A. paniculata* was evaluated. The standardised FTLEE of *A. paniculata* was given orally to mice in four groups, two of each sex (0, 300, 2000, or 5000 mg/kg BW). Body weight, poisoning symptoms, and/or death were tracked for 14 days after therapy. Animals were put to death at day 15, their internal organs were severely examined, and blood samples were taken for haematological and clinical biochemistry tests. The findings demonstrated that all of the treated animals lived, and no obvious side effects were noticed over the course of the trial. Extensive necropsy examination of all the standardised FTLEE-treated animals showed no lesions in any organs. Despite the fact that substantial changes in BUN, lymphocytes, neutrophils, hematocrit, and haemoglobin were seen, these changes were not harmful side effects of the therapy. Therefore, we came to the conclusion that there are no significant acute toxicological effects from a single oral administration of the standardised FTLEE of *A. paniculata* with an upper fixed dosage of 5000 mg/kg BW (Worasuttayangkurn *et al.*, 2019). Assessment of cellular toxicity of study samples such as *A. paniculata*, diethyl phthalate, DPAgNPs, and DPAuNPs showed minimal toxicity, while a DP gold nanoparticle showed 87.9% cytotoxicity to the cell line at a concentration of 100 µg/mL. While diethyl phthalate and DPAgNPs exhibited 90.13 and 89.68% of their toxicity at 100 µg/mL in L929 (mouse fibroblast cell line).

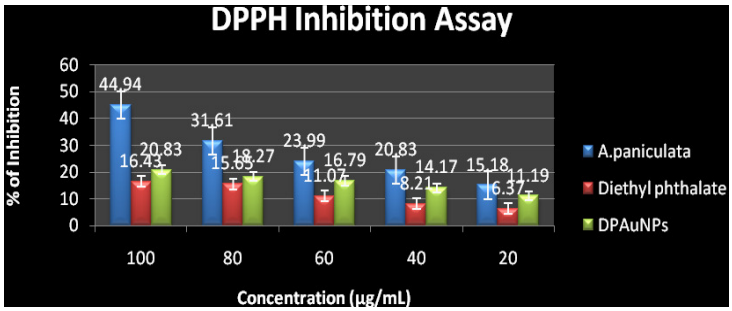

Fig. 1. Inhibitory percentage of study samples against DPPH radicals.

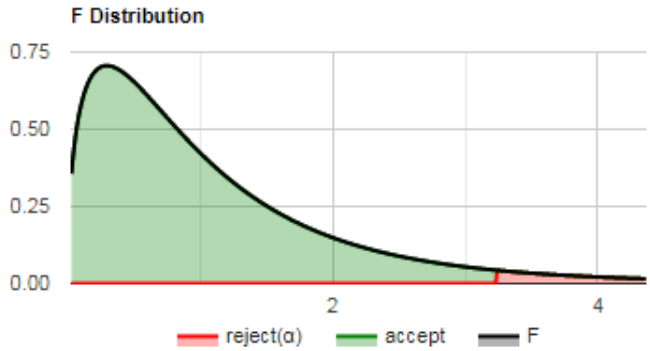

Fig. 2. One-tailed ANOVA of F-distribution of Sample groups

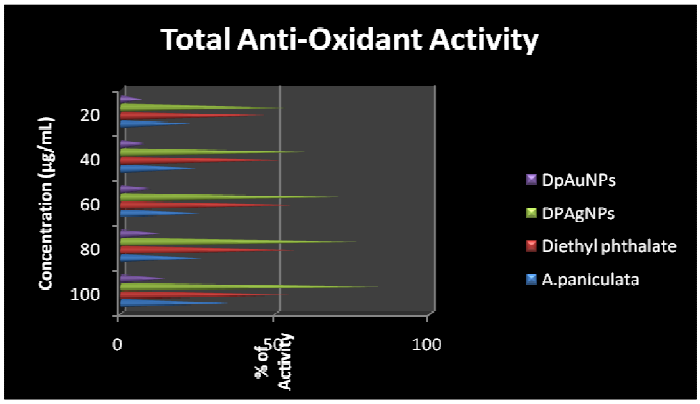

Fig. 3. Total Anti-oxidant activity by Reduction of Phosphomolebdenum

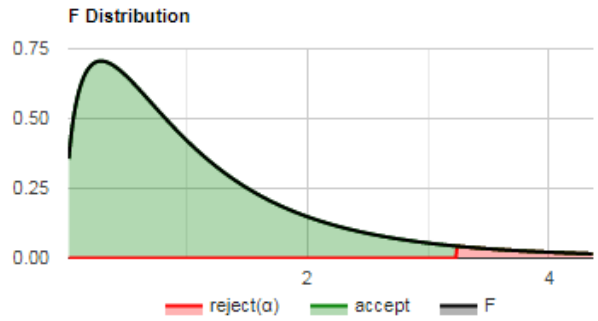

Fig. 4. One-tailed ANOVA of F-distribution of Sample groups.

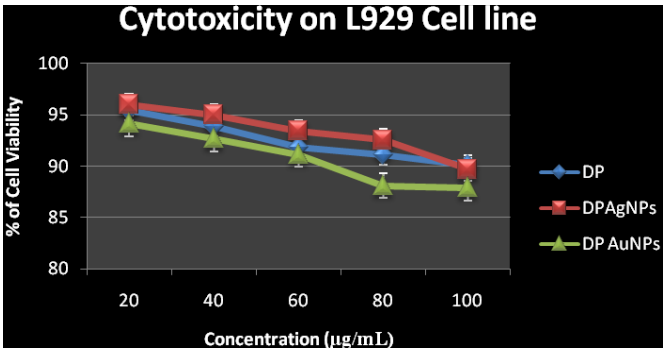

Fig. 5. Cellular toxicity effect of study samples on L929 cell line by MTT assay.

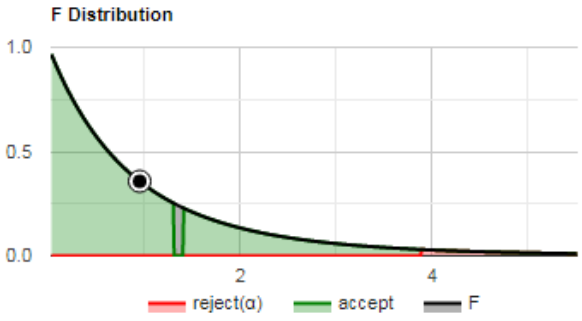

Fig. 6. One-tailed ANOVA of F-distribution of Sample groups for Cytotoxicity effects on L929 cell line.

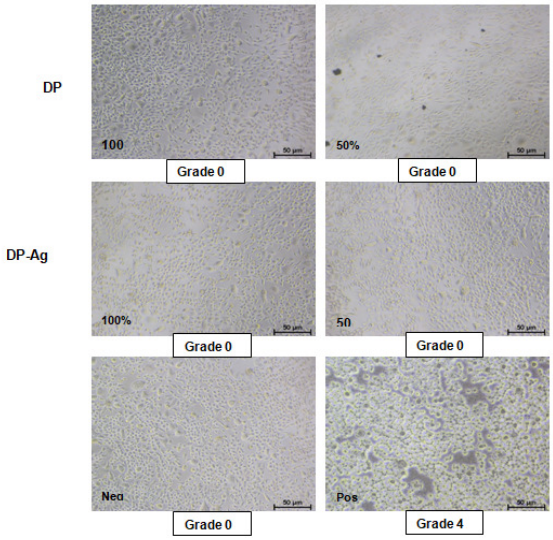

Fig. 7. Microscopic examination of cellular Anti-proliferation (Cytotoxity) on L929 cell line of Diethyl phthalate and Diethyl phthalate Silver nanoparticles.

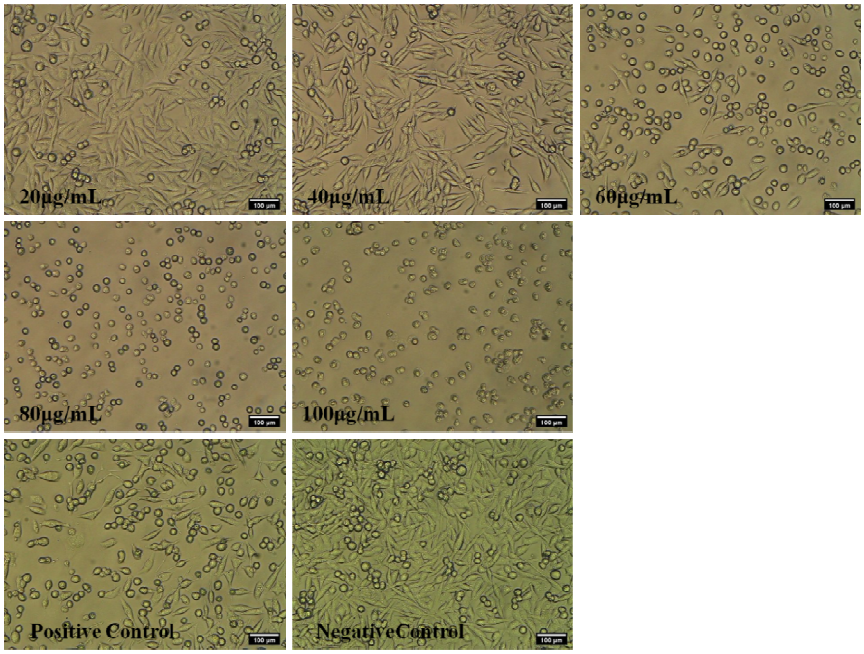

Fig. 8. Microscopic examination of cellular Anti-proliferation (Cytotoxity) on L929 cell line of Diethyl phthalate Gold nanoparticles

Table 1: Inhibitory percentage of study samples against DPPH radicals.

Concentration (µg/mL)	*A. paniculata*	Diethyl phthalate	DPAgNPs	DPAuNPs
100	44.94 ± 0.001	16.43 ± 0.001	60.36 ± 0.003	20.83 ± 0.001
80	31.61 ± 0.002	15.65 ± 0.001	56.9 ± 0.001	18.27 ± 0.001
60	23.99 ± 0.004	11.07 ± 0.001	53.51 ± 0.001	16.79 ± 0.001
40	20.83 ± 0.001	8.21 ± 0.002	51.13 ± 0.001	14.17 ± 0.001
20	15.18 ± 0.004	6.37 ± 0.001	47.62 ± 0.001	11.19 ± 0.001
IC 50	124.73	341.04	35.13	351.41

Table 2: One-tailed ANOVA of Sample groups for DPPH scavenging activity.

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	5390.2231	1796.741	37.7456	1.738e-7
Error (within groups)	16	761.6213	47.6013		
Total	19	6151.8444	323.7813		

Table 3: Total Anti-oxidant activity by Reduction of Phosphomolebdenum.

Concentration (µg/mL)	*A.paniculata*	Diethyl phthalate	DPAgNPs	DpAuNPs
100	34.78 ± 0.01	57.56 ± 0.011	83.64 ± 0.040	14.29 ± 0.1
80	26.17 ± 0.001	56.85 ± 0.002	76.6 ± 0.015	12.42 ± 0.1
60	25.49 ± 0.001	55.57 ± 0.003	71.01 ± 0.015	10.56 ± 0.1
40	24.55 ± 0.003	51.86 ± 0.003	60.04 ± 0.015	8.7 ± 0.1
20	22.75 ± 0.003	49.88 ± 0.002	54.45 ± 0.015	6.83± 0.1
IC 50	539.38	17.52	8.93	591.01

Table 4: One-tailed ANOVA of Sample groups for Total anti-oxidant activity.

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	10488.5862	3496.1954	76.176	1.089e-9
Error (within groups)	16	734.341	45.8963		
Total	19	11222.9271	590.6804		

Table 5: Cellular toxicity effect of study samples on L929 cell line by MTT assay.

Concentration (µg/mL)	DP	DPAgNPs	DP AuNPs
20	95.42 ± 0.011	95.98 ± 0.008	94.15 ± 0.011
40	93.85 ± 0.011	94.97 ± 0.002	92.67 ± 0.002
60	91.84 ± 0.005	93.41 ± 0.013	91.15 ± 0.002
80	91.13 ± 0.005	92.51 ± 0.009	88.12 ± 0.003
100	90.13 ± 0.008	89.68 ± 0.004	87.9 ± 0.002

Table 6: One-tailed ANOVA of Sample groups for Cytotoxicity effects on L929 cell line.

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	2	16.3634	8.1817	1.3557	0.2946
Error (within groups)	12	72.4206	6.0351		
Total	14	88.784	6.3417		

CONCLUSIONS

Nowadays, modern pharmacologists have turned to traditional and classic medicinal approaches for deriving multi-potential bioactive compounds to encounter a wide range of diseases and physiological disorders. In this research, the anti-oxidant potential and cytotoxic effects of a crude ethanolic extract of *Andrographis paniculata*, diethyl phthalate, silver, and gold nanoparticles synthesised using diethyl phthalate were evaluated, and the outcome revealed the improved anti-oxidant activity achieved from DPAgNPs compared with other test samples against both radicals (DPPH and phosphomolebdenum). It was also demonstrated that DPAgNPs had a less toxic effect on the L929 cell line among the study samples. The data show that using DPAgNPs for developing drug carrier systems is far superior to using *A. paniculata*, diethyl phthalate, and DPAuNPs alone, resulting in safe and fine activities.

FUTURE SCOPE

In future, in vivo toxicology studies can be performed to further investigate its toxicity. Further investigations to analyse its biocompatibility of Diethylphthalate with silver nanoparticle as drug carrier system and clinical trials are necessary for discovery of new drugs formulations.

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Conflict of Interest. None.

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