4

Results and Discussion

Medicinal plants have gained huge attention due to their importance in biological and therapeutic properties. Medicinal plants are used in ages in various traditional practices, especially for cancer treatment. Several drugs available in the market for cancer treatment are not fully effective and are associated with prolonged toxicity (Shukla and Mehta, 2015). The bioactive phytoconstituents derived from plant products are found to be highly potent in cancer research. Phytoconstituents such as saponins, lupeol, flavonoids, resveratrol, curcumin, genistein, allyl sulfide, gingerol, lycopene, berberine, indole-3-carbinol, bromelain and polyphenols are found to be highly preventive and therapeutic against cancer (Kumari *et al.*, 2018).

Cancer, a life threatening disease, has become a common ailment nowadays due to the vast changes in the life style of people and it accounts for second major cause for mortality in the world (http://www.cancer.gov). Existing anticancer treatments include chemotherapeutic drugs, radiations and surgery, but the side effects associated with these treatments are much more than the targeted treatment for cancer (Wang *et al.*, 2018).

Nanotechnology has the potential to offer solutions to the current obstacles in cancer chemotherapy. Nanoparticles can cross over the biological barriers and effective therapeutic concentration can be achieved in tumor cells with less dosage of drug administration and protect the normal tissues from toxic effect. Nano Oncology a separate branch of Nanoscience deals with the ways and means of developing alternative potentials to manage the dreadful disease like cancer (Shanmugasundaram *et al.*, 2017).

Among all the metal nanoparticles, silver nanoparticles have drawn the attention of researchers due to their widespread applications in various fields, particularly in the field of Biomedicine. The biomedical applications of silver nanoparticles include antimicrobial, antifungal, antiviral and anti-angiogenic agent are now emerging as a potential therapeutic agent for cancer treatment (Abdelghany *et al.*, 2017).

With these known matter of concern, the present research focused on **"Antioxidative and Antitumorigenic Potential of PEG Functionalized Silver Nanoparticles from Ethanolic Extract of *Volkameria inermis* Leaves to EAC Cells by *in vitro* and *in vivo* Studies"** was carried out in five phases. The first four phases dealt with *in vitro* studies and the last phase comprised of *in vivo* study.

In the first phase, preliminary screening of phytoconstituents and evaluation of antioxidative potential of *Volkameria inermis* leaves by DPPH, superoxide, hydroxyl, hydrogen peroxide and nitric oxide scavenging assays and FRAP assay were carried out in the different extracts namely petroleum ether, chloroform, ethylacetate, ethanol and water.

In the second phase, the synthesis of silver nanoparticles, functionalization of synthesized silver nanoparticles using PEG and their characterization using UV visible spectrophotometer, TEM, EDX, XRD, FTIR and Zeta potential techniques were studied.

For any drug to be used for mankind the biocompatible nature of the drug plays a key role. Hence, the biocompatible nature of the functionalized silver nano particles towards human red blood cells and the drug releasing profile of the functionalized silver nano particles at different pH conditions such as 7.4, 6.8 and 5.5 were studied in the third phase.

The fourth phase deals with the *in vitro* study namely apoptotic activity. The viability of cells was studied using MTT assay and the type of cell death was studied using AO/EtBr staining technique and DNA fragmentation was carried out using laddering pattern. Cell cycle analysis was studied by flow cytometry to find out the effect of PEG functionalized silver nanoparticles at different stages of cell cycle.

Fifth phase comprises the cytotoxicity study using trypan blue dye exclusion method and the *in vivo* antioxidative role against the standard silymarin and the antitumorigenic effect in EAC cells induced Swiss albino mice.

The results of the study are furnished and discussed under the following headings:

In vitro Studies

PHASE I

4.1 PHYTOCHEMICAL CONSTITUENTS, ANTIOXIDATIVE POTENTIAL OF DIFFERENT EXTRACTS AND CHARACTERIZATION OF ETHANOLIC EXTRACT OF *Volkameria inermis* Leaves

4.1.1 Phytochemical Constituents of Different Solvent Extracts

4.1.2 Antioxidative Potential of Different Solvent Extracts of *Volkameria inermis* Leaves

4.1.2.1 The 2, 2'- Diphenyl- 1-Picryl-Hydrazyl (DPPH) radical scavenging activity

4.1.2.2 Superoxide (O_2^-) radical scavenging activity

4.1.2.3 Hydroxyl radical ($^{\cdot}OH$) scavenging activity

4.1.2.5 Hydrogen peroxide (H_2O_2) scavenging activity

4.1.2.5 Nitric oxide (NO) scavenging activity

4.1.3 Ferric Reducing Antioxidant Power (FRAP) activity

4.1.4 Characterization of Ethanolic Extract of *Volkameria inermis* by HPLC and HPTLC Techniques

4.1.4.1 Analysis of ethanolic extract of *Volkameria inermis* by HPLC

4.1.4.2 Analysis of ethanolic extract of *Volkameria inermis* by HPTLC

PHASE II

4.2 OPTIMIZATION OF THE SYNTHESIS OF SILVER NANOPARTICLES (AgNPs), IT'S FUNCTIONALIZATION (PEGylated AgNPs) AND THEIR CHARACTERIZATION

4.2.1 Synthesis of Biologically Active AgNPs from Ethanolic Extract of *Volkameria inermis* Leaves

4.2.1.1 Formation of AgNPs

4.2.1.2 Yield of AgNPs

4.2.2 Synthesis of Functionalized AgNPs

4.2.3 Characterization of AgNPs and PEGylated AgNPs

4.2.3.1 Spectral analysis of biologically active AgNPs and PEGylated AgNPs

4.2.3.2 Transmission Electron Microscopy (TEM)

4.2.3.3 Energy Dispersive X-ray spectroscopy (EDX)

4.2.3.4 X- ray Diffraction (XRD)

4.2.3.5 Fourier Transform Infrared spectroscopy (FTIR)

4.2.3.6 Zeta potential

PHASE III

4.3 BIOCOMPATIBILITY ROLE AND DRUG RELEASING PROFILES OF PEGylated AgNPs

4.3.1 Effect of PEGylated AgNPS on the Extent of Hemolysis

4.3.1.1 Effect of PEGylated AgNPs on the Morphological Changes of Human Blood Cells

4.3.3 Drug Releasing Profiles of PEGylated AgNPs at Different pH

PHASE IV

4.4 APOPTOTIC EFFECT OF PEGYLATED AgNPs ON EAC CELL LINE

4.4.1 Cytotoxic Effect of PEGylated AgNPs to EAC Cell Line by MTT Assay

4.4.2 Effect of PEGylated AgNPs on the Morphological Changes in EAC Cell by AO/EtBr Dual Staining

4.4.3 Effect of PEGylated AgNPs on DNA Fragmentation in EAC Cell Line

4.4.4 Effect of PEGylated AgNPs on Cell Cycle Analysis by Flow Cytometry

***In vivo* Studies**

PHASE V

4.5 *In vivo* ANTIOXIDATIVE AND ANTITUMORIGENIC ACTIVITY OF PEGYLATED AgNPs IN EAC INDUCED SWISS ALBINO MICE

4.5.1 Effect of PEGylated AgNPs on Antitumorigenic Activity to EAC Cells

4.5.2 Effect of PEGylated AgNPs on the Mortality Rate of EAC Cells Induced Mice

4.5.3 Effect of PEGylated AgNPs on the Activities of Liver Marker Enzymes in Serum of EAC Challenged Swiss albino Mice

4.5.3.1 Effect on aspartate transaminase (AST)

4.5.3.2 Efect on alanine transaminase (ALT)

4.5.3.3 Effect on alkaline phosphatase (ALP)

4.5.4 Effect of PEGylated AgNPs on the Activities of Enzymic Antioxidants

4.5.4.1 Effect on catalase (CAT)

4.5.4.1 Effect on superoxide dismutase (SOD)

4.5.4.3 Effect on glutathione peroxidase (GPx)

4.5.5 Effect of PEGylated AgNPs on the Levels of Non Enzymic Antioxidants

4.5.2.1 Effect on Vitamin A

4.5.2.2 Effect on Vitamin E

4.5.2.3 Effect on reduced glutathione (GSH)

4.5.6 Effect of PEGylated AgNPs on the Rate of Lipid Peroxidation

4.5.7 Effect of PEGylated AgNPs on the Histological Status of Hepatocytes of Control and EAC Challenged Swiss albino Mice

In vitro Studies

PHASE I

4.1 PHYTOCHEMICAL CONSTITUENTS, ANTIOXIDATIVE POTENTIAL OF DIFFERENT EXTRACTS AND CHARACTERIZATION OF ETHANOLIC EXTRACT OF *Volkameria inermis* Leaves

In this phase, preliminary screening of bioactive constituents present in the different extracts namely petroleum ether, chloroform, ethyl acetate, ethanol and water in the leaves of *Volkameria inermis* and their effective scavenging potential by DPPH, superoxide, hydroxyl radical, hydrogen peroxide and nitric oxide scavenging assays and FRAP assay were carried out.

4.1.1 Phytochemical Constituents of Different Solvent Extracts

Medicinal plants are the richest bioresource for modern drugs and play a vital role in the synthesis of nanoparticles. The active phytoconstituents present in these medicinal plants make it a potential source for therapeutic aids. This bioactive molecule enhances the synthesis of nanoparticles by acting as a reducing and capping agent. The presence of

these phytoconstituents in the different extracts of *Volkameria inermis* leaves was identified by their qualitative phyto screening and the results are showed in Table 1.

Table 1

Phytochemical constituents of *Volkameria inermis* leaves in different extracts

S.No	Phytochemical Constituents	Petroleum ether	Chloroform	Ethyl acetate	Ethanol	Water
1	Alkaloids	+	-	+	+	+
2	Flavonoids	+	+	+	+	+
3	Phenols	+	+	-	+	+
4	Steroids	+	-	+	+	+
5	Saponins	+	-	+	+	+
6	Terpenoids	-	-	-	+	-
7	Tannins	-	+	-	+	-
8	Glycosides	+	+	+	+	+
9	Amino acids	-	-	-	+	+
10	Proteins	-	-	-	+	+

(+) = Presence, (-) = Absence

Among the various phytoconstituents, the bioactive molecules such as alkaloids, flavonoids, phenols, steroids, saponins, terpenoids, tannins, glycosides, amino acids and proteins were screened for their presence in the different extracts of *Volkameria inermis* leaves. Petroleum ether extract showed the presence of alkaloids, flavonoids, phenols, steroids, saponins and glycosides. Chloroform extract showed the presence of flavonoids, phenols, tannins and glycosides. However, the screening of bioactive constituents in ethyl acetate extract showed the presence of alkaloids, flavonoids, steroids, saponins and glycosides. Whereas, in ethanolic extract positive observations were found for alkaloids, flavonoids, phenols, steroids, saponins, terpenoids, tannins, glycosides, amino acids and proteins. In water extract alkaloids, flavonoids, phenols, steroids, saponins, glycosides, amino acids and proteins were found.

Of all the extracts screened for phytoconstituents, the ethanolic extract of *Volkameria inermis* leaves showed maximum bioactive constituents, followed by water extract. The therapeutic properties of medicinal plants are attributed to the presence of various bioactive constituents such as alkaloids, flavonoids, glycosides, vitamins, tannins, and coumarins. These constituents physiologically interact with human system and interfere with pathogens, interrupt their growth and maintain the body in a disease free state (Mittal *et al.*, 2014).

Varughese and Tirupathi (2013) identified the bioactive compounds such as tannins, phlobatinins, phenols, carbohydrates, proteins, flavonoids, saponins, alkaloids, steroids, terpenoids, triterpenoids and cardiac glycosides in dried powders of ripe and unripe fruits of *Aegle marmelos*. The dried powders were subjected to different solvent extractions such as methanol, ethanol and acetone. The study showed a similar positive observation of both polar and non-polar phytoconstituents such as tannins, phenols, carbohydrates, proteins, flavonoids, alkaloids, steroids, terpenoids and triterpenoids in the ethanolic extract of unriped fruit.

4.1.2 Antioxidative Potential of Different Solvent Extracts of *Volkameria inermis* Leaves

Antioxidants act as "free radical scavengers". Free radicals are continuously generated in living systems and in exogenous environment that can cause oxidative damage to unsaturated fatty acids in membranes, thiol groups in proteins and to the nucleic acid bases in DNA and RNA. These damages change the cellular components and accelerate aging process and cause various diseases such as coronary heart diseases, cancer, inflammation, decline in immune system, neurological diseases and atherosclerosis. These harmful free radicals are neutaralized by antioxidants (Dawidowicz and Olszowy, 2011). *In vitro* antioxidative potential of different solvent extracts of *Volkameria inermis* was evaluated by assessing their ability to scavenge DPPH, superoxide, hydroxyl radical, hydrogen peroxide, nitric oxide and FRAP against the standard antioxidant ascorbic acid.

4.1.2.1 The 2, 2'- Diphenyl- 1-Picryl-Hydrazyl (DPPH) radical scavenging activity

The DPPH is commonly used to evaluate the free radical scavenging activity of antioxidants. It is a stable free radical which accepts an electron or hydrogen radical to form a stable diamagnetic molecule. The antioxidants induce the dose dependent reducing ability of DPPH radical as indicated by the decrease in absorbance at 517 nm (Lagnika *et al.*, 2011).

Figure 6

DPPH scavenging activity of different extracts of *Volkameria inermis*

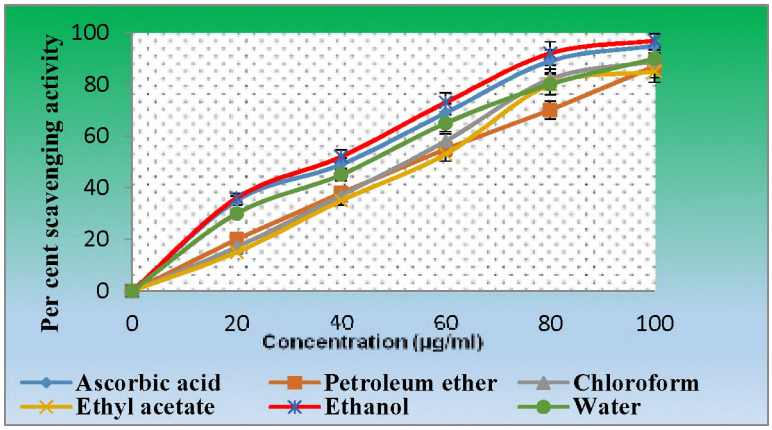


Figure 6 clearly indicates the dose dependent increase in the scavenging activity of different extracts of *Volkameria inermis* leaves. The IC_{50} values of ascorbic acid, petroleum ether, chloroform, ethyl acetate, ethanol and water were found to be 42.0, 58.0, 54.0, 56.0, 38.0 and 46.0 µg/ml respectively. The reductants present in different extracts of *Volkameria inermis* leaves exert antioxidant action by breaking the free radical chain reactions by donating a hydrogen atom. This increased scavenging activity in ethanolic extract may be due to the synergetic role as indicated by the minimum IC_{50} value 38.0 µg/ml which was found to be more effective than that of standard ascorbic acid.

Dechayont *et al.* (2017) showed that the ethanolic leaf extract of *Pogostemon cablin* resulted the highest dose dependent antioxidant activity against DPPH with IC_{50} at 18±0.90 µg/ml and ABTS with IC_{50} at 20±0.24 µg/ml. Abdel-Hameed *et al.* (2012) reported DPPH scavenging activity of the methanolic extracts of *Conocarpus erectus* leaves, stems, fruits and flowers. Similarly, dose dependent DPPH scavenging effect was

observed in *Syzygium cumini* ethanolic seed extract against the standard antioxidant ascorbic acid (Banerjee and Narendhirakannan, 2011).

4.1.2.2 Superoxide (O_2^-) radical scavenging activity

Superoxide anion is oxygen centered radical with selective reactivity. Among the free radicals, superoxide anion radical is one of the strongest reactive oxygen species, which gets converted to hydrogen peroxide and hydroxyl radical, which may induce the damage of the biomolecules and result in chronic diseases. In *in vivo* condition, superoxide radicals are formed due to the leakage of electron from the electron transport chain in mitochondria (Rajasekharan and Mahimadoss, 2016). Superoxide anion (O_2^-) is more reactive in hydrophobic environment than in aqueous environment. The protonated form of superoxide anion i.e, hydroperoxyl radical (HO_2^-) is more reactive than superoxide anion itself.

Figure 7

Superoxide scavenging activity of different extracts of *Volkameria inermis*

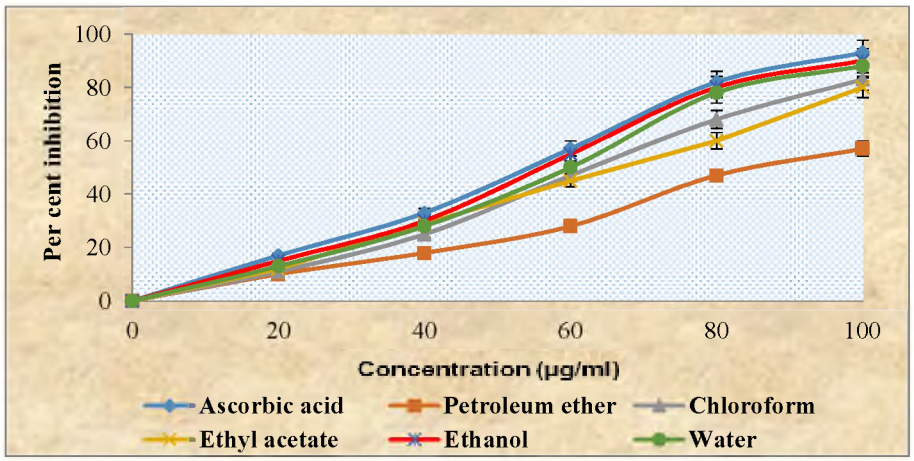


Figure 7 shows the effective superoxide scavenging activity of different extracts of *Volkameria inermis* leaves. The extracts showed a significant increase in their scavenging potential in a concentration dependent manner. The IC_{50} values of ascorbic acid, petroleum ether, chloroform, ethyl acetate, ethanol and water were found to be 54, 84, 63, 65, 56 and 60 µg/ml respectively. Ethanol extract showed IC_{50} at minimum concentration which was comparable to the standard ascorbic acid.

Overproduction of superoxide anion leads to redox imbalance, associated with harmful physiological consequences. But our study showed a dose dependent scavenging of superoxide anion to maintain redox homeostasis by inhibiting superoxide anion production.

Ujwala *et al.* (2012) reported that the alcoholic extract of *Phyllanthus amarus* showed a dose dependent inhibition of superoxide anion against the standard antioxidants curcumin and ascorbic acid.

4.1.2.3 Hydroxyl radical (˙OH) scavenging activity

Hydroxyl radical is formed by a metal catalyzed reaction through Haber–Weiss reaction or Fenton reaction. These radicals are extremely reactive and attack many cellular constituents such as lipids, nucleic acids and proteins. It is capable of damaging the biological molecules in the human body, leading to carcinogenesis (Manian *et al.*, 2008; Ramkumar *et al.*, 2009).

Figure 8

Hydroxyl radical scavenging activity of different extracts of *Volkameria inermis*

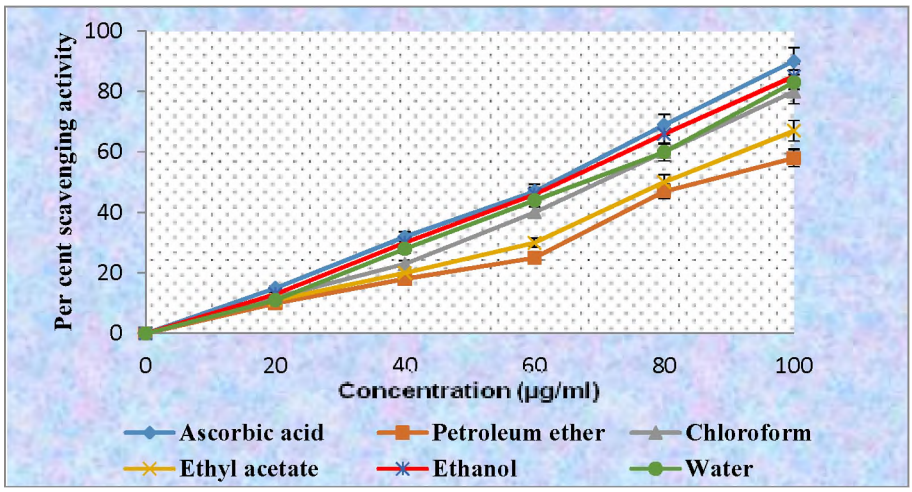


Figure 8 shows a dose dependent effect of the different extracts and ascorbic acid on the scavenging of hydroxyl radicals. The IC_{50} values of ascorbic acid, petroleum ether, chloroform, ethyl acetate, ethanol and water extracts were found to be 62, 85, 70, 79, 63 and 67 µg/ml respectively. The inhibition of hydroxyl radicals was due to the reductants

attributed by the bioactive constituents in the different extracts of *Volkameria inermis* leaves.

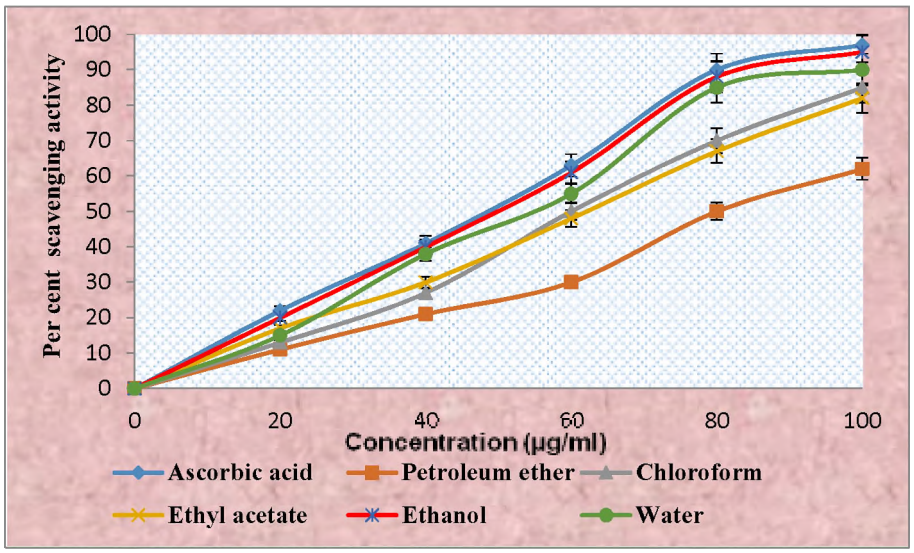
Karuna *et al.* (2018) reported that the ethanolic root extract of *Asparagus racemosus* Linn. exhibited a potent antioxidant activity against DPPH, hydroxyl and nitric oxide. The IC_{50} values were found to be 468.57±3.002 µg/ml (DPPH), 508.17±7.37 µg/ml ($^{\cdot}OH$) and 416.57±5.08 µg/ml (NO). Similar dose dependent inhibition of $^{\cdot}OH$ generation was observed by free radical scavenging activity of fruit extract of *Cucumis trigonus* (Balakrishnan and Kokilavani, 2011), *Avicennia marina* (Forssk), *Vierh pneumatophore* (Lincy *et al.*, 2012), *Dioscorea oppositifolia* tuber extract (Paulpriya and Mohan, 2012) and ethanolic extract of *Nymphaea pubescens* (Rushender *et al.*, 2012).

4.1.2.4 Hydrogen peroxide (H_2O_2) scavenging activity

Hydrogen peroxide is not a radical species but plays a role to contribute oxidative stress through Fenton reaction. Hydrogen peroxide can easily cross the cell membranes. In *in vivo* condition hydrogen peroxide reacts with iron complexes inside the cell to generate highly reactive hydroxyl radicals and which in turn causes toxic effects (Nishaa *et al.*, 2012).

Figure 9

Hydrogen peroxide scavenging activity of different extracts of *Volkameria inermis*



The scavenging activity of hydrogen peroxide in the different extracts of *Volkameria inermis* leaves was found to be increased in a dose dependent manner (Figure 9). The effective increase in the scavenging activity was due to the presence of bioactive constituents in the different extracts, which can donate electrons to the hydroxyl radical produced by H_2O_2 and neutralizes it into water (H_2O). The IC_{50} values of ascorbic acid, petroleum ether, chloroform, ethyl acetate, ethanol and water extracts in hydrogen peroxide scavenging activity were found to be 48, 80, 60, 63, 48.5 and 54 µg/ml respectively.

Akuodor *et al.* (2017) reported that the ethanolic root and bark extracts of *Salacia lchmbachii* showed significant inhibition at different concentration of the extract against DPPH radical, nitric oxide and hydrogen peroxide by scavenging activities with amino acid as standard. Okoko (2009) reported that the organic fractions of *Garcinia kola* and *Njavara rice bran* showed a dose dependent hydrogen peroxide activity. Similarly, Mudoi *et al.* (2012) resulted that the alcoholic extract of dried pulp of *Garcinia pedunculata* showed a good scavenging activity.

4.1.2.5 Nitric oxide (NO) scavenging activity

Nitric oxide radicals are produced by macrophages under inflammatory condition. Because of its mutagenic nature nitric oxide interferes with DNA and causes various carcinomas and inflammatory diseases. Nitric oxide reacts with superoxide radical and forms highly reactive metabolite peroxynitrite (ONOO–) anion which is highly toxic to humans (Shajeela *et al.*, 2012).

Figure 10

Nitric oxide scavenging activity of different extracts of *Volkameria inermis*

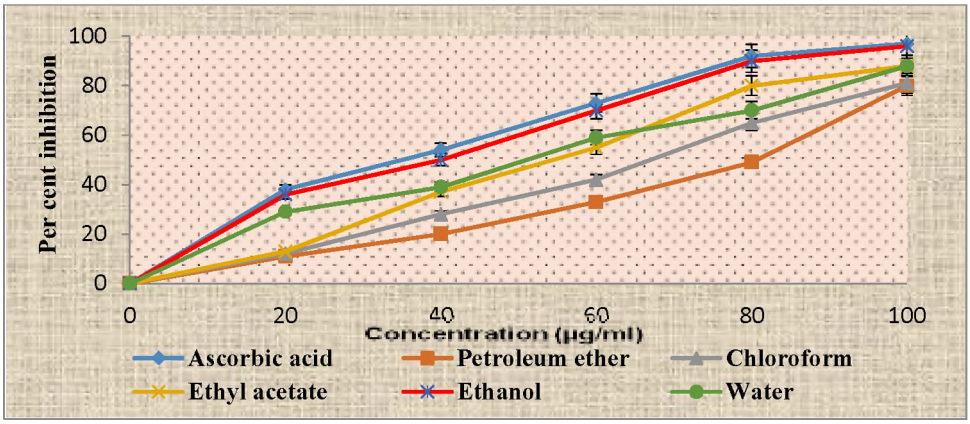


Figure 10 shows a dose dependent increase in nitric oxide scavenging activity. The IC_{50} values of ascorbic acid, petroleum ether, chloroform, ethyl acetate, ethanol and water extracts were found to be 34.0, 80.0, 66.0, 54.0, 40.0 and 50.0µg/ml respectively.

The suppression of nitric oxide was achieved by the different extracts of *Volkameria inermis* leaves due to the presence of various bioactive constituents which acted as reductants and proved as good antioxidants by decomposing the sodium nitroprusside.

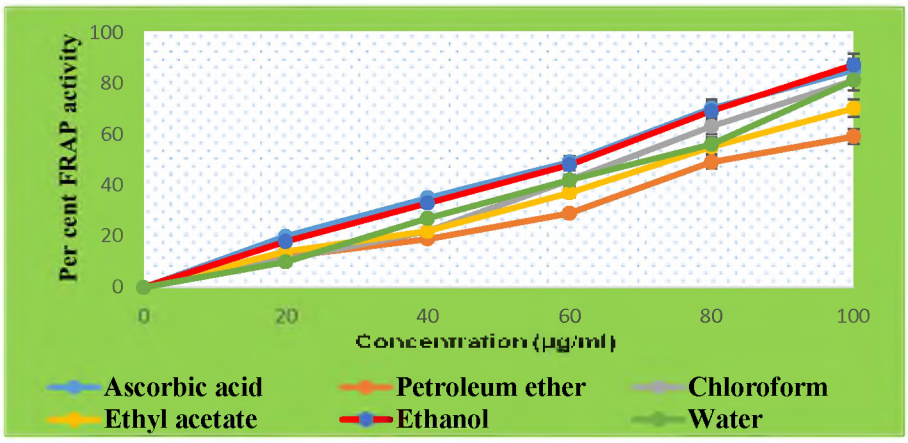
The ethanolic leaves extract of *Cnidoscolus chayamansa* showed a better *in vitro* antioxidant activity against DPPH, nitric oxide and hydroxyl assays respectively. The inhibitory concentration was found to be 262.02 µg/ml for DPPH, 343.01 µg/ml for nitric oxide scavenging activity and 224.47 µg/ml for hydroxyl scavenging activity (Pillai *et al*., 2017). Grace-Lynn *et al*. (2012) observed a dose dependent inhibition of nitric oxide in the leaf extract of *Lantana camara*. Similarly, the ethylacetate and ethanolic fraction of *Pisonia grandis* showed a scavenging of nitric oxide against the standard ascorbic acid (Jayakumari *et al*., 2012).

4.1.3 Ferric Reducing Antioxidant Power (FRAP) activity

Ferric reducing antioxidant power assay was commonly used to determine the antioxidant activity of plant extracts. The antioxidant activity was directly proportional to its reducing power (Kaneria *et al*., 2012). The FRAP values increased with increasing concentrations of the extracts as shown in Figure 11.

Figure 11

Ferric reducing antioxidant power of different extracts of *Volkameria inermis*



The IC_{50} values of ascorbic acid, petroleum ether, chloroform, ethyl acetate, ethanol and water extracts were found to be 62, 81, 68, 74, 60 and 72 µg/ml respectively. Generally, lower the IC_{50} value greater the antioxidant potential. Hence, the ethanolic extract of *Volkameria inermis* leaves showed a good antioxidant potential, compared to all the other extracts.

Nishaa *et al.* (2012) reported that the ethanolic extract of *M. arundinacea* was found to be an effective scavenger of ABTS, DPPH, H_2O_2, and NO and also possessed a good reducing power (FRAPS) activity. The antioxidant activity of the ethanolic extract of *Alstonia angustifolia* was assessed by their ability to scavenge DPPH, FRAP and H_2O_2 scavenging activities. In FRAP assay, the extract showed a good total antioxidant activity and effectively scavenged the free radicals (Rahim *et al.*, 2017). Adebiyi *et al.* (2017) reported that the ethanolic extract of stem and leaves of *Grewia carpinifolia* acts as a good antioxidant by quenching DPPH, ABTS cation decolorization test and FRAP total antioxidant power assay.

Of all the extracts used for *in vitro* free radical scavenging study, the ethanolic extract of *Volkameria inermis* leaves showed a good antioxidative potential followed by water extract. The greater antioxidative potential of ethanolic extract was correlated with the initial phytoconstituents study where the ethanolic extract showed more number of phytoconstituents.

The study was supported by previous findings. The ethanolic bark extract of *Polyalthia longifolia* exhibited maximum DPPH scavenging activity 93.4 per cent followed by the water extract which exhibited 23 per cent at a concentration of 120µg/ml (Gayathri and Jeyanthi, 2012). Similarly, Rajamurugan *et al.* (2013) reported that the ethanolic extract of *Aegle marmelos*, *Acalypha indica*, *Ocimum sanctum*, *Eclipta alba* and *Alternanthera sessilis* exhibited maximum antioxidant activity as evaluated by their reducing power assay and also the scavenging of DPPH and ABTS radicals. Kamboj *et al.* (2014) reported the better scavenging activity of ethanolic extract of leaf, stem and root of *X. strumarium* when compared to petroleum and chloroform extracts by DPPH assay. Similarly, Das and Devi (2015) showed better scavenging acitivity against DPPH, ABTS, superoxide, nitric oxide, hydroxyl and hydrogen peroxide by the ethanolic bark

extract of *Terminalia bellirica* than other extracts namely petroleum ether, chloroform and water.

The results obtained are in accordance with the above literatures cited which supported that the ethanolic extract of *Volkameria inermis* leaves acted as a good radical scavenger and an effective antioxidant agent. Hence, for the following studies the ethanolic leaves extract of *Volkameria inermis* was used.

4.1.4 Characterization of Ethanolic Extract of *Volkameria inermis* by HPLC and HPTLC Techniques

For the identification and to quantify the main constituents within the plant, chromatographic techniques such as HPLC and HPTLC are universally used (Loescher *et al.*, 2014).

4.1.4.1 Analysis of ethanolic extract of *Volkameria inermis* by HPLC

The HPLC chromatograms of the standard quercetin (Figure 12) and the ethanolic leaves extract of *Volkameria inermis* (Figure 13) were recorded and compared with each other. A peak at the retention time of 3.753 minutes in the ethanolic leaves extract correspond with the standard peak obtained at the retention time of 3.458 minutes. This observation clearly confirmed the presence of quercetin in the ethanolic extract of *Volkameria inermis* leaves. The extract also showed the presence of additional peaks, indicating the presence of other phytocompounds.

Figure 12

HPLC profile of standard quercetin

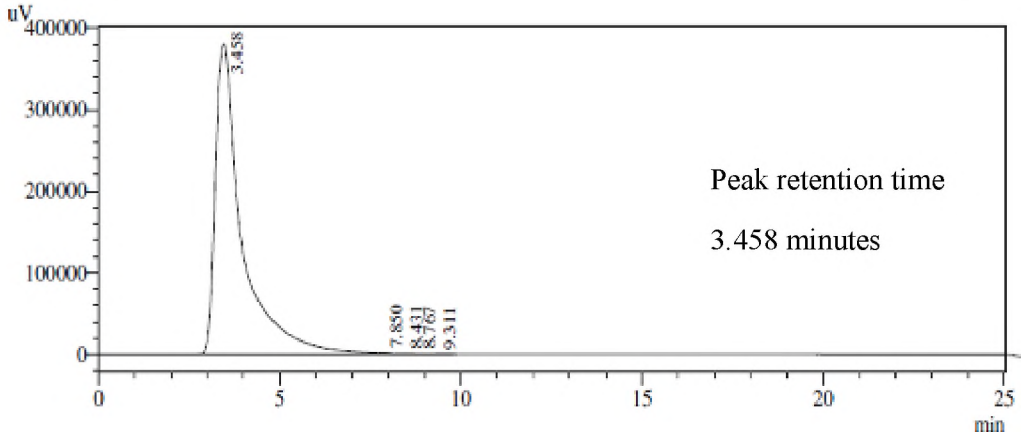
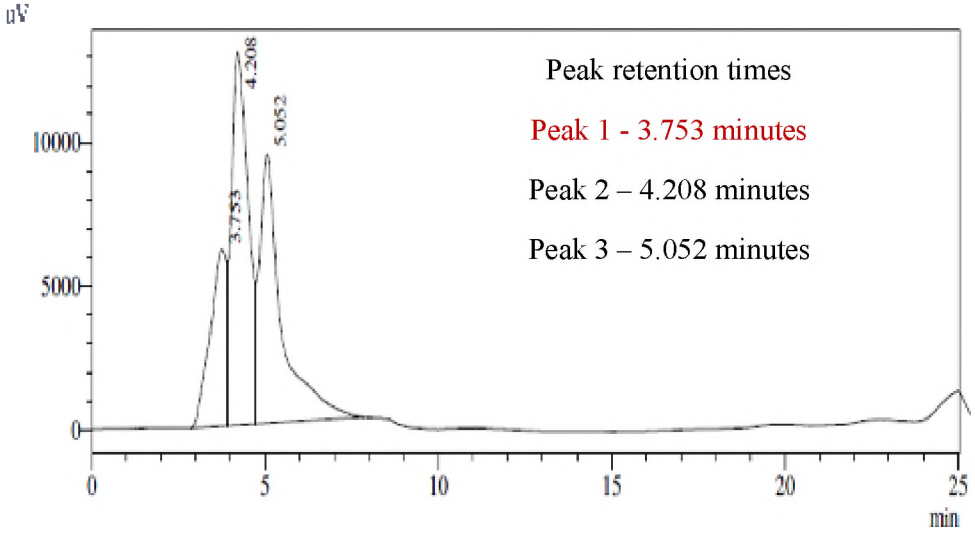


Figure 13

HPLC profile of ethanolic extract of *Volkameria inermis* leaves



In the present study the HPLC analysis showed the ethanolic leaves extract of *Volkameria inermis* contained quercetin as the major flavonoid along with the other phytoconstituents.

Quercetin is a naturally occurring flavonoid detected in more plant species, including *Carmona retusa* (Chandrappa *et al*., 2014) and *Piper* guinneensis (Bruno *et al*., 2015). Similarly, using HPLC analysis quercetin and various flavonoid components such as bergenin, quercetin, quercitrin and one isocoumarin namely isosalipurposide were reported in the ethanolic flowers extract of *Corylopsis coreana* (Seo *et al.,* 2016).

4.1.4.2 Analysis of ethanolic extract of *Volkameria inermis* by HPTLC

The HPTLC analysis was carried out to confirm the presence of flavonoid and their derivatives in the ethanolic extract of *Volkameria inermis* using quercetin as standard. The analysis of flavonoid component quercetin was found to be highly reproducible in the mobile phase chloroform: ethyl acetate: acetic acid (12:7:1). The calibration for the quercetin standard was found to be linear (Figure 16) indicating the sensitivity of the method. The HPTLC fingerprint showed the presence of quercetin as major peak in the leaves extract of *Volkameria inermis* which was confirmed by comparing the peaks of the standard with that of the sample peaks. The R_f value was found to be 0.01 comparable to the standard R_f value.

Figure 14

HPTLC profile of standard quercetin

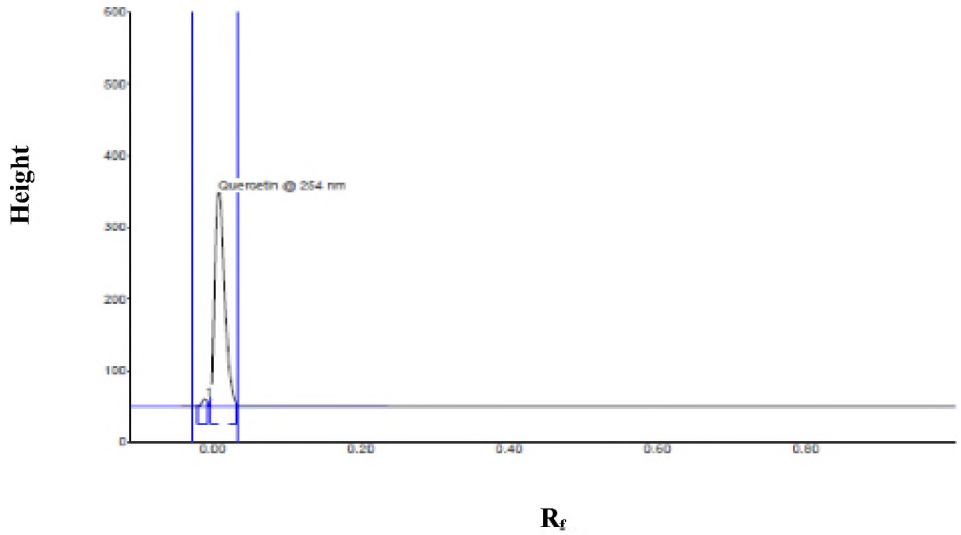


Figure 15

HPTLC profile of ethanolic leaves extract of *Volkameria inermis*

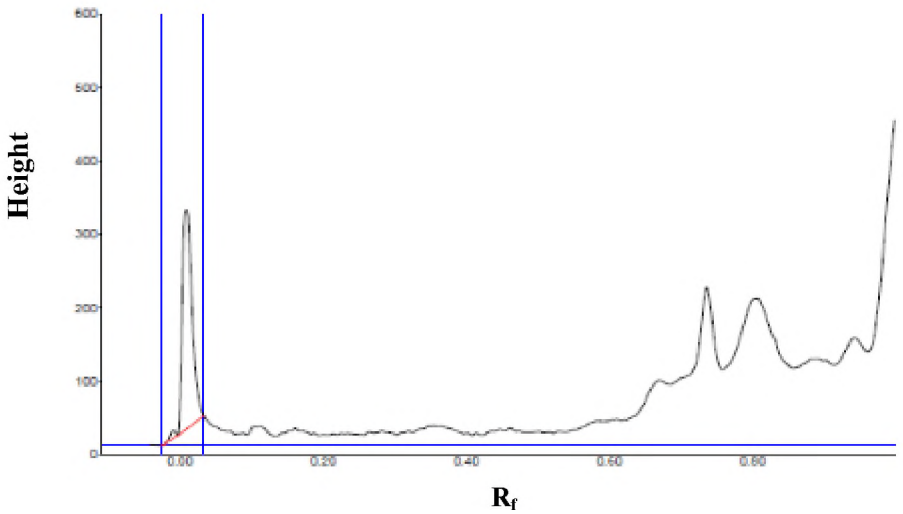


Figure 16

Calibration for standard quercetin

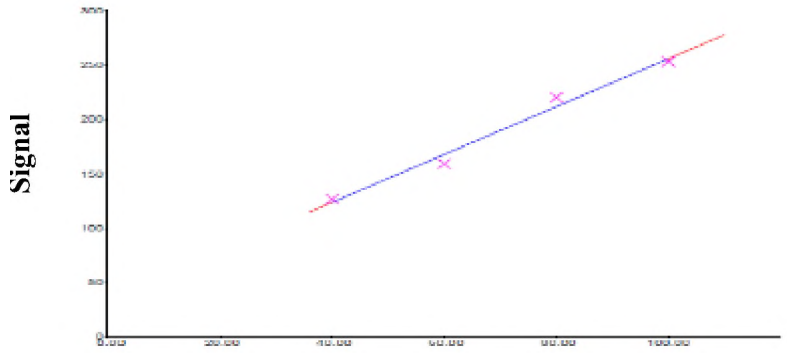
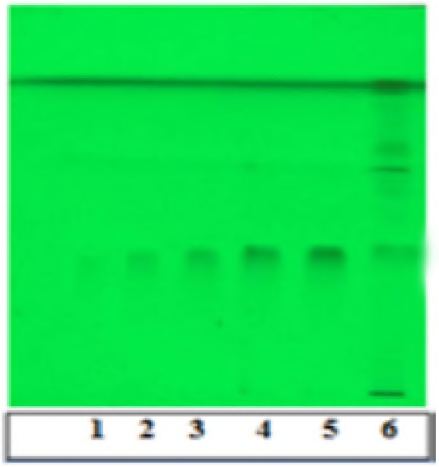


Plate 3

Visualization of quercetin band at 254 nm in HPTLC chromatogram



Lane 1- 5 = Standard quercetin of different concentration,

Lane 1 – 0.2 µg, Lane 2 – 0.4 µg, Lane 3 – 0.6 µg, Lane 4 – 0.8 µg , Lane 5 – 1.0 µg

Lane 6 = Ethanolic extract of *Volkameria inermis* leaves extract

Several authors have used HPTLC to confirm the presence of flavonoids. Lakshmi *et al.* (2012) reported the presence of quercetin and rutin in the ethanolic leaf extract of *Acacia catechu.* The presence of quercetin and other phenolic compounds in the leaves of *Barleria cristata* Linn. and *Lycopodium clavatum* were reported in HPTLC fingerprint (Narmadha and Devaki, 2012; Srivastava *et al.*, 2012).

Based on the findings of Phase I it can be concluded that the ethanolic extract of *Volkameria inermis* leaves exhibited a promising effect of free radical scavenging activity and served as an effective antioxidative agent. The findings of HPLC and HPTLC chemical fingerprints confirmed the presence of quercetin a flavonoid compound and their derivatives apart from other phytoconstituents which acted as a potent source of natural antioxidant. Knowing the antioxidative potential, synthesis of silver nanoparticles and PEG functionalized silver nanoparticles from the ethanolic extract of *Volkameria inermis* leaves and its characterization were carried in Phase II.

PHASE II

4.2 OPTIMIZATION OF THE SYNTHESIS OF SILVER NANOPARTICLES (AgNPs), IT'S FUNCTIONALIZATION (PEGylated AgNPs) AND THEIR CHARACTERIZATION

Nanotechnology an emerging field of nanoscience has raised the possibility of using therapeutic nanoparticles for biomedical applications. Nanoparticles synthesized from plants gain more importance as it was free from toxic chemicals, more reliable and more economical. Among the various metal nanoparticles silver and gold nanoparticles play a vital role in biomedical field as biomolecular recognition, biosensing and targeted drug delivery system (Roopan *et al.*, 2014). To address a nanoparticle various physicochemical metrics such as size distribution, shape, aggregation state, topology, surface charge, chemical composition and solubility has to be taken into consideration. Other factors such as their biocompatibility, target interactions and their drug release into the environment have to be addressed for their use in biomedical applications (Barnard, 2010). Various techniques were used to characterize the nanoparticles. In our research work we utilized techniques such as UV absorption spectroscopy, TEM, EDX, FTIR, Zeta potential and XRD to divulge the nature of the synthesized nanosilver and its functionalized nano using PEG from *Volkameria inermis* leaves extract, and the results arrived are discussed below.

4.2.1 Synthesis of Biologically Active AgNPs from Ethanolic Extract of *Volkameria inermis* Leaves

Different methods namely heating in microwave for 10, 20, 30 and 40 seconds, heating in water bath for 5, 10, 15 and 20 minutes at 60°C and exposure to sunlight for 5,

10, 15 and 20 minutes were carried out. The biological synthesis of silver nanoparticles occurred in all the three methods was monitored by a change in colour (qualitatively) and increase in the yield (quantitatively). The silver nanoparticles were collected from the reaction mixture by centrifugation at 13,000 rpm.

4.2.1.1 Formation of AgNPs

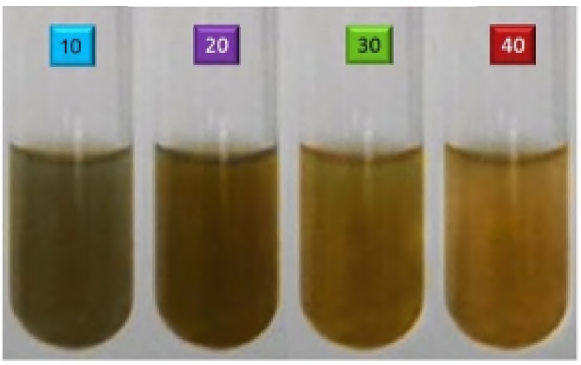
Rapid synthesis of silver nanoparticles occurred in all the three different methods namely heating in microwave, heating in water bath and exposure to sunlight. The efficiency of *Volkameria inermis* leaves extract in the biosynthesis of biologically active silver nanoparticles was confirmed by the colour change from yellow to intense brown, which was recorded by visual observation. The intensity of colour also increased in all the three different methods with the increase in the exposure time and duration of incubation.

Plate 4

Formation of biologically active silver nanoparticles from the ethanolic extract of *Volkameria inermis* leaves

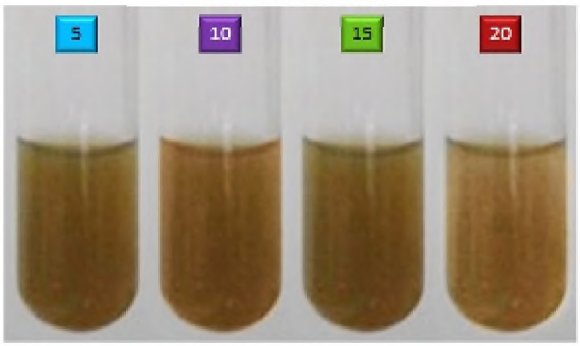
a) Microwave heating

Time in seconds



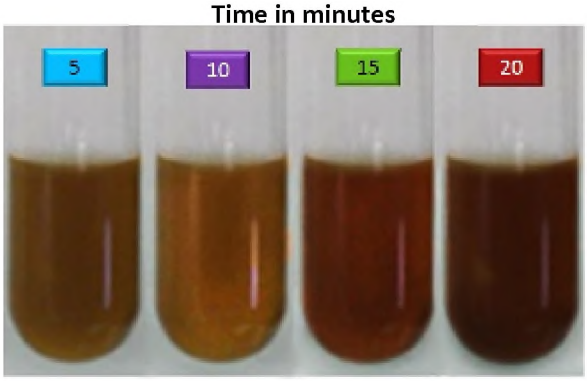
b) Water bath heating at 60°C

Time in minutes



c) Sunlight exposure

Time in minutes



The results showed that all the three different methods were efficient in the synthesis of biologically active silver nanoparticles from the ethanolic leaves extract of *Volkameria inermis* (Plate 4).

Of all the three different methods a notable increase in the intensity of colour was observed on exposure to sunlight for 5 to 20 minutes. These observations confirmed that sunlight exposure for 20 minutes was the best method for the synthesis of biologically active silver nanoparticles using the ethanolic leaves extract of *Volkameria inermis*.

Synthesis of silver nanoparticles from natural sources has gained importance in recent days. The synthesis exhibited a notable change in colour from yellow to brown and the intensity of brown colour is directly proportional to the increase in incubation period and temperature, which indicated the reduction of silver nitrate by the extract (Parveen and Rao, 2014). Kumar *et al.* (2017) reported that silver nanoparticles synthesized from aqueous extract of *Prunius persica* at room temperature showed a colour change from yellow to intense brown.

Enormous reports are obtainable on the green synthesis of silver nanoparticles using plant parts as environment friendly substitutes to chemical methods. The fruit extract of *Terminalia chebula* (Edison and Sethuraman, 2012), *Dillenia indica* (Singh *et al.*, 2013), *Emblica officinalis* (Ramesh *et al.*, 2015) and *Solanum lycopersicums* (Umadevi *et al.*, 2013) were established by the development of brown colour.

Several researchers have reported that, extract of banana, neem and black tulsi leaves showed a change in colour from yellowish brown to colloidal brown under microwave heating (Banerjee *et al.*, 2014). Similarly, Alam *et al.* (2015) have reported that synthesis of silver nanoparticles by incubating in water bath at 80°C for 4 hours showed a colour change from yellowish to brown colour. A rapid synthesis of silver nanoparticles was obtained by exposure to sunlight using the bark extract of *Cochlospermum religiosum* (Sasikala *et al.*, 2014). Under sunlight exposure the extract of *Ficus racemosa* (Tetgure *et al.*, 2015) and *Ziziphora tenuior* (Sadeghi and Gholamhoseinpoor, 2015) leaves showed a rapid synthesis of silver nanoparticles. Abalaka *et al.* (2014) and Brahmachari *et al.* (2014) also reported the same results in various plants.

4.2.1.2 Yield of AgNPs

Followed by the intensity of colour change, the yield in the silver nanoparticles synthesized from the leaves of *Volkameria inermis* was estimated. Table 2 lists the yield of silver nanoparticles synthesized by various methods from *Volkameria inermis* leaves extract. The synthesis was found to be increased in all the methods with the increase in the duration of exposure. Conversely, there was a marked increase in the nanoparticle synthesis pattern from *Volkameria inermis* leaves under sunlight exposure, which showed the maximum yield at 20 minutes.

Table 2

Yield of biologically active silver nanoparticles from ethanolic extract of *Volkameria inermis* leaves

S.No	Method	Duration of exposure	Yield (mg) from 100 ml
1	Heating in microwave	10 seconds	9 ± 0.07
		20 seconds	13 ± 0.1
		30 seconds	18 ± 0.05
		40 seconds	20 ± 1.03
2	Heating in water bath at 60°C	5 minutes	23 ± 1.07
		10 minutes	27 ± 2.9
		15 minutes	29 ± 2.03
		20 minutes	30 ± 2.09
3	Exposure to sunlight	5 minutes	35 ± 1.03
		10 minutes	38 ± 1.09
		15 minutes	42 ± 3.39
		20 minutes	**45 ± 5.07**

The synthesis of biologically active silver nanoparticles from the ethanolic extract of *Volkameria inermis* leaves extract using the three different methods, an intense colour change and increase in yield was more pronounced in sunlight exposed samples than that of the other methods, reiterating that this was found to be the best method among the methods tested.

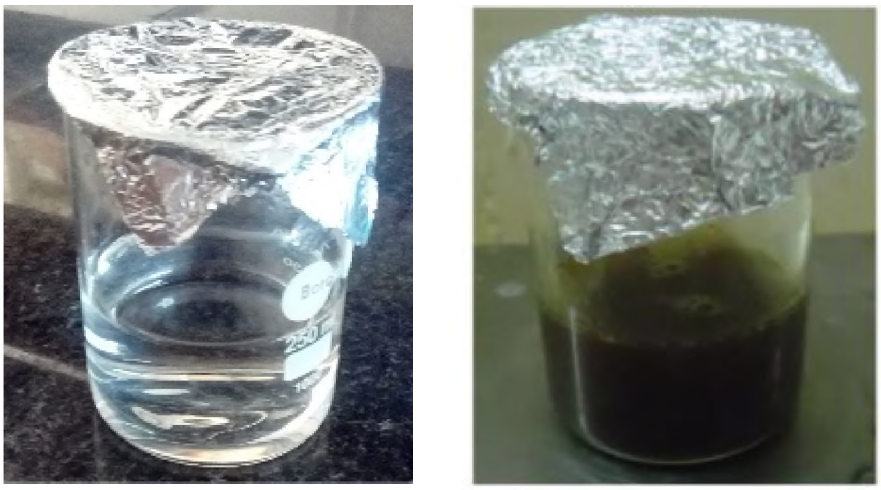
The synthesis of nanoparticles using light was found to be highly advantageous than chemical synthesis method (Grzelczak and Marzan, 2014). Prasad (2015) have reported that synthesis of nanoparticles under sunlight exposure method was found to be the best method for the synthesis of nanoparticles. Thus, our results also showed that exposure to sunlight generated rapid and high yield in silver nanoparticles than the other methods, which is an inexpensive, potential and the best method for the synthesis of silver nanoparticles.

4.2.2 Synthesis of Functionalized AgNPs

Followed by the synthesis of AgNPs, its functionalization was carried out using PEG-4000. Figure 17 shows the suspension of PEG with the biologically active AgNPs by sunlight exposure method for 20 minutes.

Figure 17

Synthesis of PEGylated AgNPs



a) PEG solution b) PEG suspension with AgNPs

The stability of the synthesized biologically active AgNPs was increased by its functionalization using PEG – 4000 which prevented the aggragation of the AgNPs (Chang *et al.*, 2016). The successful capping of PEG with AgNPs was confirmed by the following characterization studies.

4.2.3 Characterization of AgNPs and PEGylated AgNPs

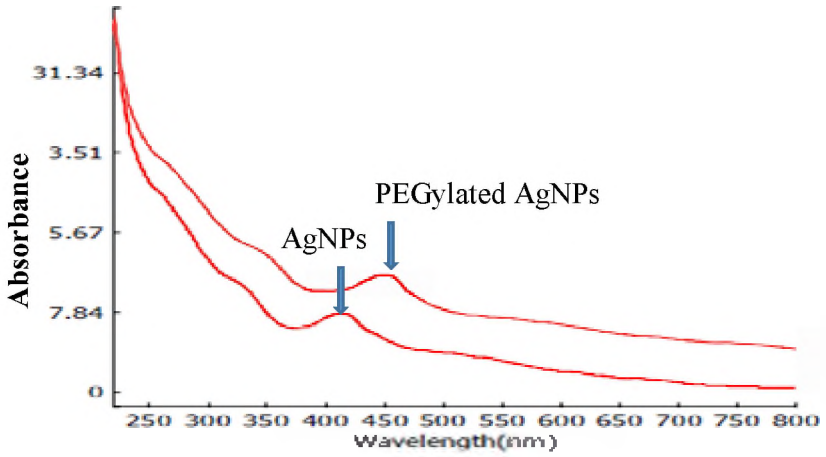
Among the different methods used for the synthesis of silver nanoparticles, the maximum intensity of colour observation and yield was observed in twenty minutes exposure to bright sunlight. Hence, this method was used for the synthesis of silver nanoparticles for further study and characterization.

4.2.2.1 Spectral analysis of biologically active AgNPs and PEGylated AgNPs

The optical properties of the synthesized AgNPs and PEGylated AgNPs were analyzed by UV- visible spectroscopy. The silver nanoparticles synthesized from *Volkameria inermis* leaves extract showed a distinct peak at 430 nm, which are characteristic for AgNPs (Figure 18).

Figure 18

Absorption spectrum of AgNPs and PEGylated AgNPs



***Absorbance units as recorded in Shimadzu-Bio Spec-nano, Japan**

The absorption band formed is influenced by the size, shape, composition, morphology, distribution and stability of the synthesized nanoparticles (Joseph and Mathew, 2014). Roy *et al.* (2017) reported that the silver nanoparticles synthesized using *Azardirachta indica* leaves extract was centered at 430 nm. Similarly, characteristic peak at 430nm has been reported using *Lactobaciluus acidophilus* 01 strain (Namasivayam *et al.*, 2011). Silver nanoparticles synthesized from *Andrographis*

paniculata showed an absorption band at 410 nm (Pannerselvam *et al.*, 2011). Stiufiuc *et al.* (2013) reported that the silver colloids synthesized by reduction of silver nitrate using short chain of PEG showed an absorption band at 433 nm, where $AgNO_3$ was added dropwise to PEG solution.

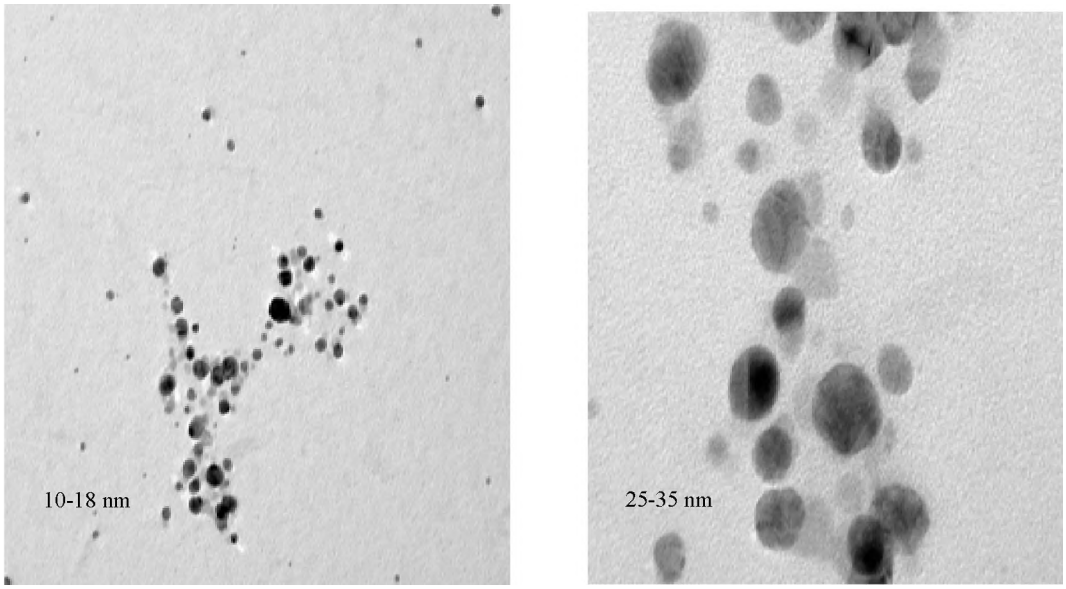
4.2.2.2 Transmission Electron Microscopy (TEM)

The morphology and size of the synthesized AgNPs and PEGylated AgNPs were analyzed by TEM. The AgNPs and PEGylated AgNPs synthesized from *Volkameria inermis* leaves extract showed spherical shape (Plate 5). The size of the AgNPs and PEGylated AgNPs were 10-18 nm and 25-35 nm in size respectively. The nanoparticles were monodispersed without aggregation. The size of the AgNPs and PEGylated AgNPs were within the nanometer scale (≤ 100 nm), which clearly depicted that the synthesized AgNPs and PEGylated AgNPs were within the nano scale.

Plate 5

TEM image of AgNPs and PEGylated AgNPs

i) AgNPs **ii) PEGylated AgNPs**



The morphology and size of the synthesized AgNPs and PEGylated AgNPs were clearly studied using TEM. In our research work the TEM analysis effectively showed that both the AgNPs and PEGylated AgNPs were well dispersed, spherical in shape and are ideally fitted for therapeutic applications. The synthesis of silver and gold

nanoparticles from the extract of *Erigeron annus* (L.) pers flower extract showed spherical shape with size ranging from 15 to 60 nm and 20 to 100 nm (Velmurugan *et al.*, 2014). In another study the average diameter of the silver nanoparticle synthesized from *Cynodon dactylon* under sunlight exposure was found to be 8-10 nm (Sahu *et al.*, 2013). Syu *et al.* (2014) reported that the nanoparticles synthesized from *Arabidopsis* plant extract under sunlight exposure showed spherical, decahedral and triangular shapes with a size of 8-47 nm.

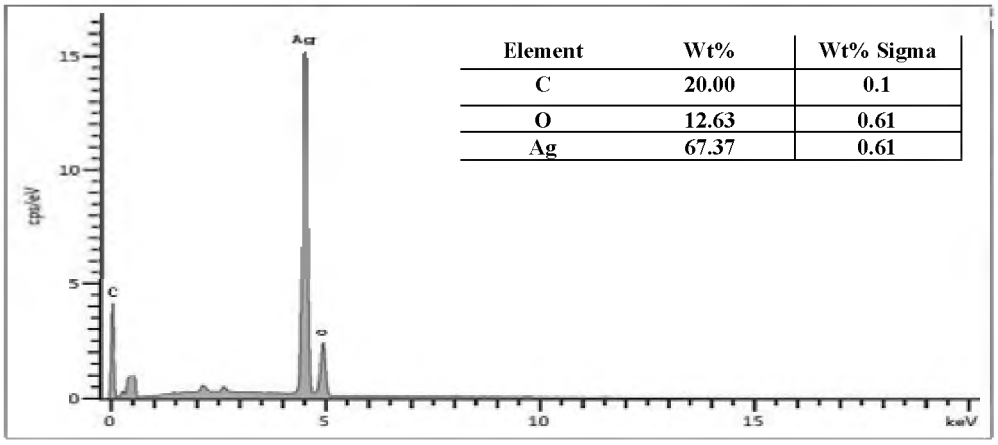
4.2.2.3 Energy Dispersive X- ray spectroscopy (EDX)

The EDX is a reliable tool to determine the elemental composition of the synthesized AgNPs and PEGylated AgNPs from *Volkameria inermis* leaves extract. The results of EDX profile of the AgNPs and PEGylated AgNPs from *Volkameria inermis* leaves extract showed the presence of typical peaks for silver and the percentage of silver was found to be 67.37 and 64.04 in AgNPs and PEGylated AgNPs respectively (Figure 19 and Figure 20).

Additional peaks were also observed which indicated the presence of carbon and oxygen, representing the existence of organic compounds in the AgNPs and PEGylated AgNPs.

Figure 19

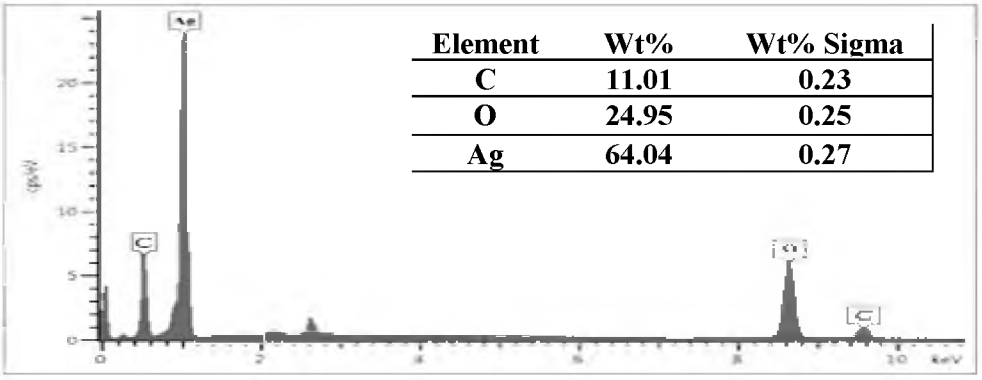
EDX composition of AgNPs



Element	Wt%	Wt% Sigma
C	20.00	0.1
O	12.63	0.61
Ag	67.37	0.61

Figure 20

EDX composition of PEGylated AgNPs



Element	Wt%	Wt% Sigma
C	11.01	0.23
O	24.95	0.25
Ag	64.04	0.27

The EDX technique is used to determine the elemental analysis and quantitative determination of available minerals in the nanoparticles (Laloy *et al.*, 2014). EDX spectra of the synthesized AgNPs and PEGylated AgNPs indicated the presence of carbon, oxygen and silver, which evidenced the conjugation of organic molecules in the synthesis of nanoparticles. Geetha *et al.* (2012) reported that silver nanoparticles formed from the leaf extract of *Chromolaena odorata* showed the presence of silver signals on EDX analysis. The EDX profile of the AgNPs synthesized from *Ocimum tenuiflorum*, *Azadirachta indica* and *Musa balbisiana* showed the presence of carbon and oxygen (Priya *et al.*, 2014). Similar results were noted in the AgNPs synthesized from the aqueous fruit extract of *P.Peruviana* (Rashid and Sabir, 2014), tea leaves extract (Sun *et al.*, 2014), leaves of *Cichorium intybus* and *Boerhaavia diffusa* (Bharathi *et al.*, 2014) indicating the consistency of EDX measure. Bonnia *et al.* (2018) reported that silver nanoparticles synthesized from aqueous leaves extract of *I. cylindrical* showed the presence of elemental silver. Moreover, Rafi Shaik *et al.* (2018) also reported that silver nanoparticles synthesized from aqueous leaves extract of *Origanum vulgare* and confirmed the presence of elemental silver.

4.2.2.4 X- Ray Diffraction (XRD)

The phase purity and the crystallinity of the biosynthesized AgNPs and PEGylated AgNPs from the *Volkameria inermis* leaves extract were examined by XRD technique. The XRD profiles of the synthesized AgNPs and PEGylated AgNPs are depicted in Figure 21 and Figure 22. The diffraction peaks at 38.12°, 44.313°, 64.464° and

77.424° could be indexed to (111), (002), (022) and (113) planes of face centered cubic structure of AgNPs. The peaks observed at 11.043°, 28.238°, 29.913° are indexed at (110), (111) and (200) planes are due to the presence of PEG moieties. This confirmed the presence of silver and PEG and the highly crystalline nature of the particles. The pattern of AgNPs and PEGylated AgNPs synthesized from *Volkameria inermis* leaves extract showed some additional peaks, which might be due to the presence of organic molecules in the extract. Thus, the results of X-ray diffraction pattern corroborated with our EDX profile results, which validated the presence of other molecules that facilitate the synthesis of nanoparticles.

Figure 21

XRD pattern of AgNPs

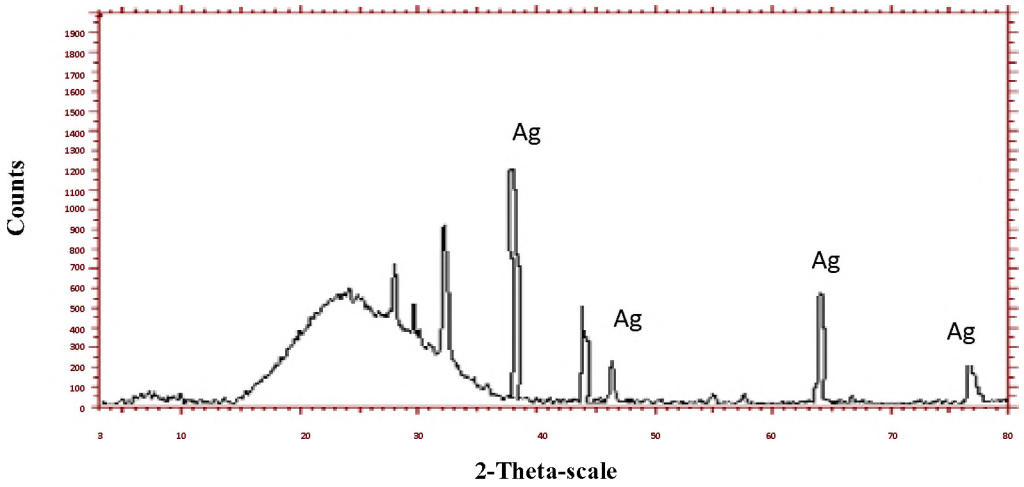
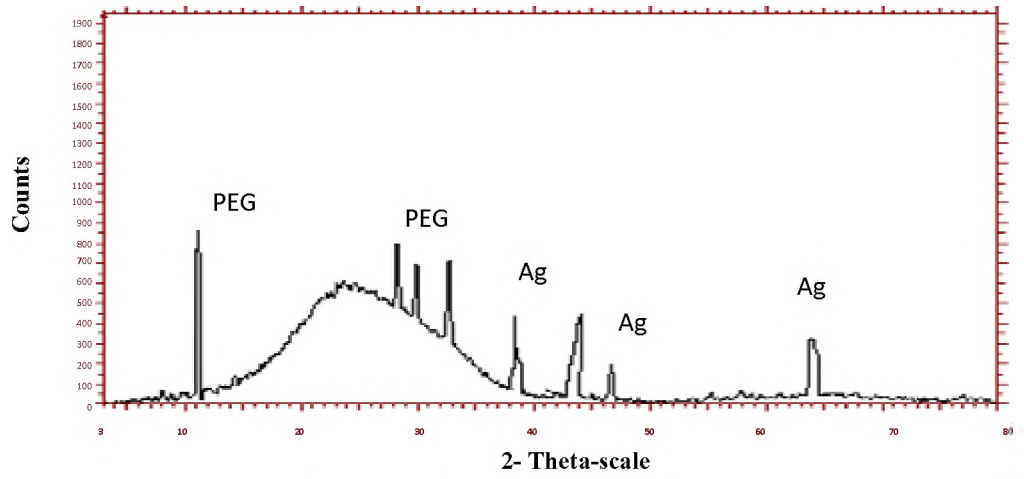


Figure 22

XRD pattern of PEGylated AgNPs



The XRD technique is used to identify the crystalline nature by comparing the obtained pattern with the reference library and assigning its nanocrystalline nature (Das *et al.*, 2010). In the present research work, the XRD patterns of the silver and PEG coated silver reaffirmed the presence of silver, PEG coated on the surface of silver and other materials in the synthesized nanomaterials. It also proved the crystalline nature of the bioactive nanoparticles. The XRD analysis of silver nanoparticles synthesized from *Coriandrum sativum* leaves extract showed the presence of elemental silver peaks (Khan *et al.*, 2018). Thakur *et al.* (2013) reported that the silver nanoparticles from *Acacia arabica* are highly crystalline in nature. The XRD pattern for the silver nanoparticles synthesized from *Terminalia* bellirica showed typical peaks for silver (Anand and Mandal, 2015). Another researcher, Rathi Sre *et al.* (2015) also showed that the silver nanoparticles synthesized from the aqueous root extract of *Erythrina indica Lam* showed typical peaks for silver. The presence of PEG moiety was noted in the nanocomposites of silver / chitosan / polyethylene glycol (Ahmad *et al.*, 2011).

All these reports supported our results for the bioactive nanoparticles and confirmed the crystalline nature.

4.2.2.5 Fourier Transform Infrared spectroscopy (FTIR)

The FTIR is an important tool to determine the functional groups responsible for the reduction of silver nitrate to silver nanoparticles and stabilization of the nanoparticles (Lakshmi *et al.*, 2015). The FTIR spectrum was documented for the silver nanoparticles (AgNPs) and PEGylated AgNPs of ethanolic leaves extract of *Volkameria inermis*. The FTIR spectra (Figures 23,24 and 25) of *Volkameria inermis* leaves extract showed the characteristic peaks of OH, aromatic and alkene groups which may be involved in the reduction and stabilization of silver nanoparticles and PEGylated silver nanoparticles synthesized from the leaves extract.

Figure 23

FTIR spectrum of *Volkameria inermis* leaves extract

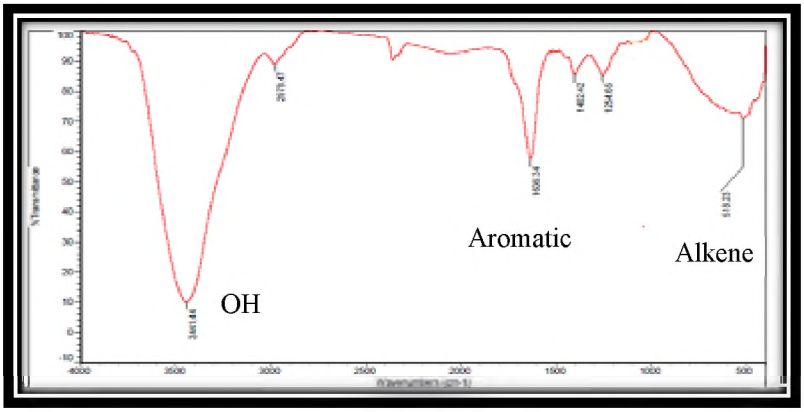


Figure 24

FTIR spectrum of AgNPs

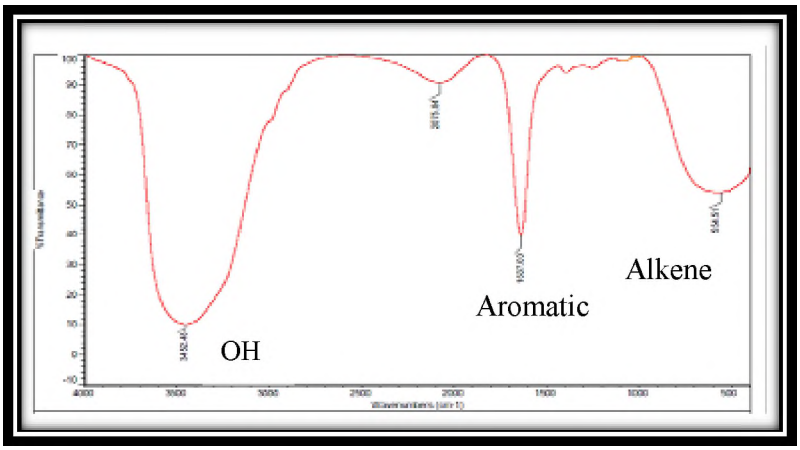
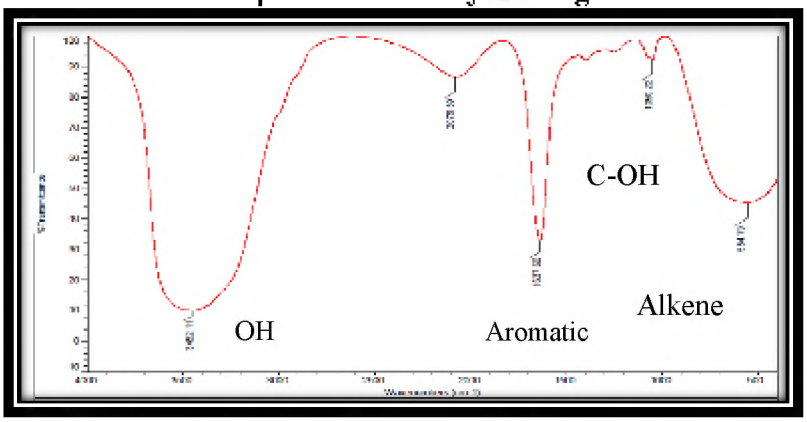


Figure 25

FTIR spectrum of PEGylated AgNPs



The presence of typical functional groups such as OH, aromatic and alkene in the extract may be acted in the capping and as stabilizing agent for the synthesis of silver nanoparticles and PEG coated silver nanoparticles. In addition the FTIR spectrum of PEGylated AgNPs showed C-OH bond which successfully coated PEG on the surface of AgNPs.

The silver nanoparticles synthesized from *Annona muricata* leaf extract showed the presence of phytoconstituents namely amides, alkenes, aliphatic amines, alkanes and alkyls, responsible for the reduction of silver nitrate (Santhosh *et al.*, 2015). The FTIR spectrum of silver nanoparticles synthesized from the fruit extract of European black elderberry (*Sambucus nigra*) revealed the presence of quinoidal, and ·OH of polyphenol (David *et al.*, 2014). Subarani *et al.* (2013) reported that the FTIR spectra of the AgNPs synthesized from *Vinca rosea* emphasized the functional groups such as alkenes, amine and carboxylic acids involved in the synthesis.

Several research have been conducted for the functionalization of nanoparticles. The PEG coated iron oxide (Fe_2O_3) nanoparticle showed an absorption band, representing the stretching vibration of alcoholic hydroxyl C-OH bond. Which, strongly supported that PEG was successfully coated on Fe_2O_3 nanoparticles (Kavithaa *et al.*, 2016).

Our results are in accordance with the above studies and showed the presence of several functional groups in *Volkameria inermis* leaves extract which were expressed in the synthesized silver nanoparticles and PEGylated silver nanoparticles, possibly rendering capping and stabilization of the particles.

4.2.2.6 Zeta potential

The surface charge of the synthesized silver nanoparticles and the functionalized silver nanoparticles were determined by means of zeta potential measurements. The physicochemical stability and interaction of biomolecules with nanoparticles can be studied. The zeta potential values of the biosynthesized AgNPs and PEGylated AgNPs were found to be at 18.5 and -25.0 mV (Figure 26 and Figure 27). These values were found to be within the normal range (-30 to + 30).

Figure 26

Surface charge analysis of AgNPs by Zeta potential

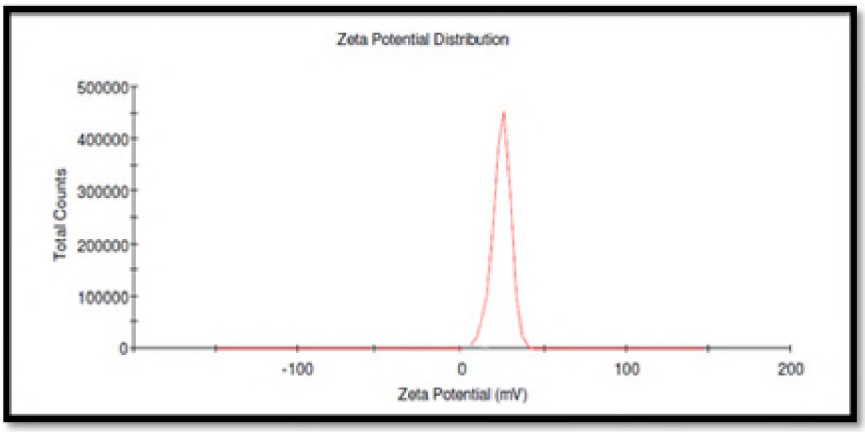
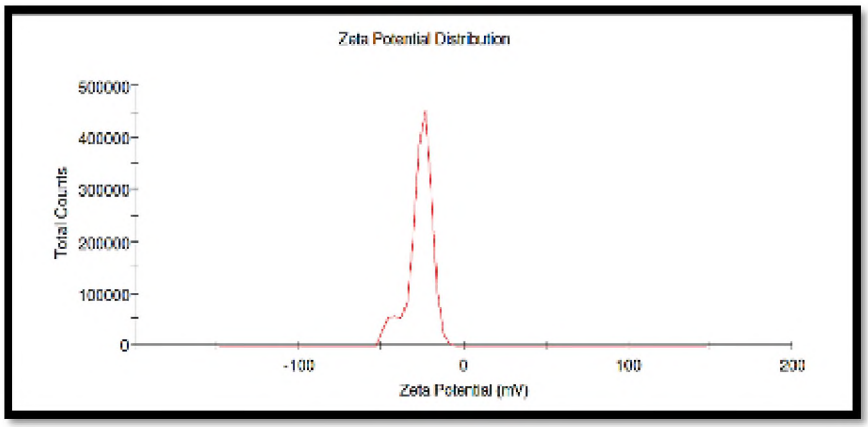


Figure 27

Surface charge analysis of PEGylated AgNPs by Zeta potential



The hydroxyl group (OH) of PEG act as the covering entity, which coat the surface of silver nanoparticles which are positively charged. Colloidal stabilization of silver and PEG coated silver occur due to the presence of Van der Waals force which exist between the negatively charged oxygen group in PEG and the positively charged group which encompasses on the surface of silver nanoparticles (Karakoti *et al.*, 2011).

The zeta potential of silver nanoparticles synthesized from the stem bark extract of *Callicarpa maingayi* was equal to 35.5 ± 3. 7 mV which strongly indicated the stability of silver nanoparticles due to electrostatic repulsion (Shameli *et al.*, 2012). Similarly, Bhatia *et al.* (2011) reported that the nanoparticles prepared from the ethanolic extract of *Ziziphus mauritiana* leaves showed a zeta potential of 30mV. The metallic nanoparticle

prepared by means of chemical synthesis method showed a positive zeta potential of + 20 mV indicated the stability of the nanoparticle (Kavithaa *et al.*, 2016). Mandal *et al.* (2012) reported that PEG functionalized silver nanoparticles by chemical reduction method revealed a zeta potential of -21.7 mV. Similarly, PEG coated palladium nanoparticles synthesized by chemical synthesis method showed a negative zeta potential of -32.8 mV (Shanthi *et al.*, 2015).

The results obtained in our study clearly showed that the negatively charged PEGylated AgNPs gets attached to the positively charged silver nanoparticles by Van der Waals force of attraction. Thus indicating the stability of the synthesized colloidal PEGylated AgNPs.

Phase II study concluded that, all the different techniques used to characterize AgNPs and the PEGylated AgNPs showed the successful capping of phytoorganic components on silver and PEG coated silver, to form well dispersed, spherical nanoparticles with the size ranging from 10-18nm and 25-35nm respectively. The functional groups present on their surface confirmed their bioactive potential and showed a good stability profile with a net positive charge on the surface of AgNPs and a net negative charge on the surface of PEGylated AgNPs. The ideally suited structures for both AgNPs and PEGylated AgNPs were successfully characterized. Among which, the PEG coated AgNPs was chosen for further studies due to their increased circulation time and improved hydrophilicity nature. Hence, the ability of the PEG coated AgNPs as a drug carrier and its biosafety was assessed by tracing their drug release profiles at different pH conditions and its biocompatibility with human red blood cells. The results of these studies were presented in the next phase.

PHASE III

4.3 BIOCOMPATIBILITY ROLE AND DRUG RELEASING PROFILES OF PEGylated AgNPs

The synthesis of nanomaterials from plant based sources are found to be non-toxic and safe for human use. Among all the nanomaterials, metallic nanoparticles have drawn the attention of scientists due to its evocative physical and chemical properties. Nanomaterials have various biomedical applications such as diagnostics, therapeutics and targeted drug delivery. Functionalization of metallic nanoparticles using surfactants such

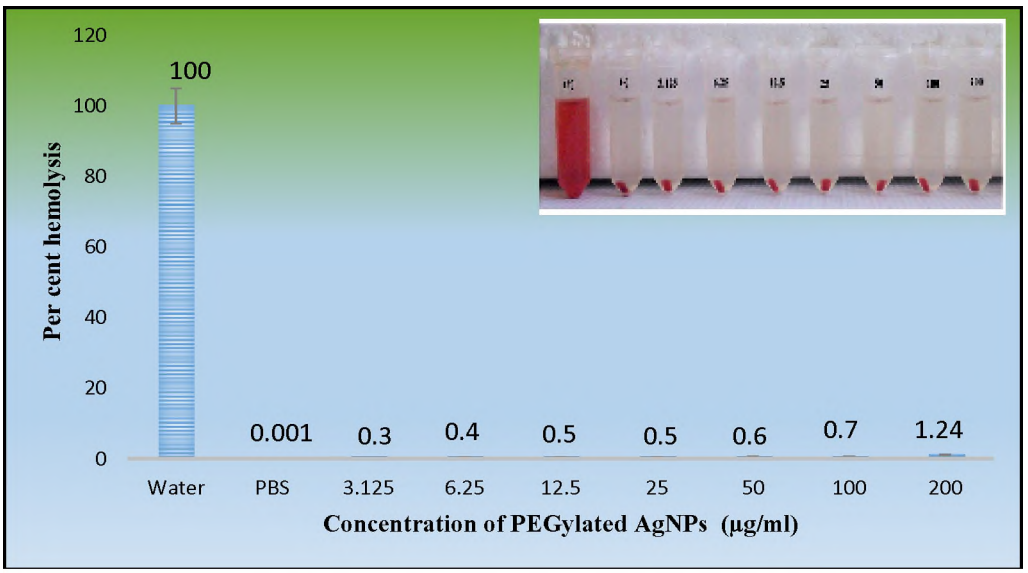
as polymers improves the stability, enhances the circulation time and increases the therapeutic efficiency (Kumar *et al.*, 2013). Hence, it is imperative to study the biocompatible nature and drug release profiles of PEGylated AgNPs. Macromolecules and particles are uptaken by cells via endocytosis mechanism. Endocytic pathway leads to the formation of membrane vesicles known as endosomes and lysosomes which have slightly acidic environment. Besides, some tumor sites have slightly acidic environment (Wong *et al.*, 2011). With this known matter of concern, we aimed to synthesize a pH sensitive PEGylated AgNPs as an anticancer drug for Ehrlich Ascites Carcinoma cells. The drug release response was evaluated under different pH conditions 7.4, 6.8 and 5.5 towards human red blood cells. The pH 7.4 corresponds to the environment of blood. pH 6.8 indicates tumor tissue pH and pH 5.5 represents mature endosomes of tumor cells at 37°C, since this is close to physiological temperature.

4.3.1 Effect of PEGylated AgNPS on the Extent of Hemolysis

The toxicity study of PEGylated AgNPs synthesized from *Volkameria inermis* leaves was tested on human red blood cells from healthy volunteers to determine the biocompatibility. Hemoglobin release analysis (Figure 28) showed the hemolytic control and PEGylated AgNPs at different concentrations. When water was added to RBCs hemolysis takes place and the released hemoglobin was measured. This serves as the positive control and represents 100 per cent hemolysis. RBCs suspended with PBS showed 0.001 per cent hemolysis, which was taken as the negative control. When, PEGylated AgNPs was added the percentage of hemolysis was found to be less than 5 per cent comparable to the negative control. It has been reported that upto 5 per cent hemolysis was permissible for biomaterials (Singhal and Roy 2002). The largest percentage of hemolysis at the tested concentration was found to be 1.24 ± 0.04 per cent for 200µg/ml. Since this is much lower than 5 per cent PEGylated AgNPs was considered as hemocompatible for drug delivery applications.

Figure 28

Effect of PEGylated AgNPs on hemolysis at different concentration



The values are mean ± SD of triplicates

4.3.1.1 Effect of PEGylated AgNPs on the Morphological Changes of Human Blood Cells

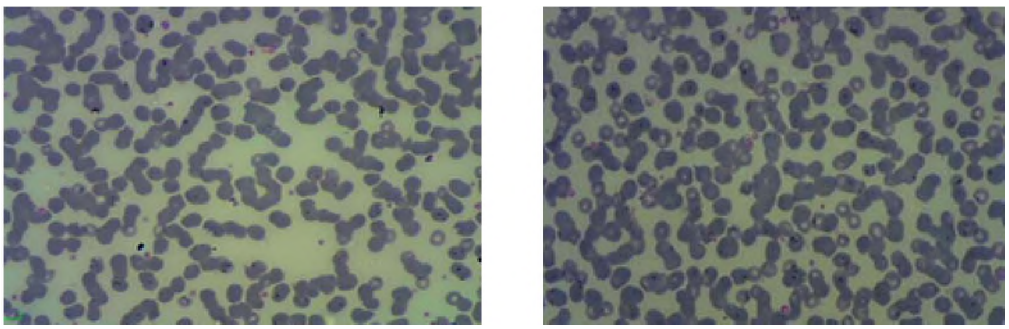
The non-toxicity of the synthesized PEGylated AgNPs was further investigated based on the changes in the morphology of human red blood cells incubated with 200µg/ml of PEGylated AgNPs. The morphology of the red blood cells was observed using an inverted microscope and the photographs are shown in Plate 6.

The cell morphology analysis showed no change in morphology of red blood cells when compared with that of the control. Thus, the cell morphology analysis was corroborated with that of hemoglobin release analysis signifying the biocompatible nature of PEGylated AgNPs.

Plate 6

Effect of PEGylated AgNPs on the morphology of human RBC (200 µg /ml)

A) Human RBC-Control (40 X) **B) Human RBC+ PEGylated AgNPs (40 X)**



In our study, the absence of hemolysis was due to the surface modification of AgNPs by the biocompatible polymer PEG which prevented the adhesion of both the nanoparticles and the red blood cells. Thus, this simple surface modification stratagem ensures the safety of PEGylated AgNPs for biomedical applications.

The PEGylated AgNPs synthesized from the leaves of *Volkameria inermis* did not exhibit any toxicity to the red blood cells in the *in vitro* toxicity tests (hemolysis and cell morphology) studied. These observations clearly showed that the PEGylated AgNPs are biocompatible for the human system.

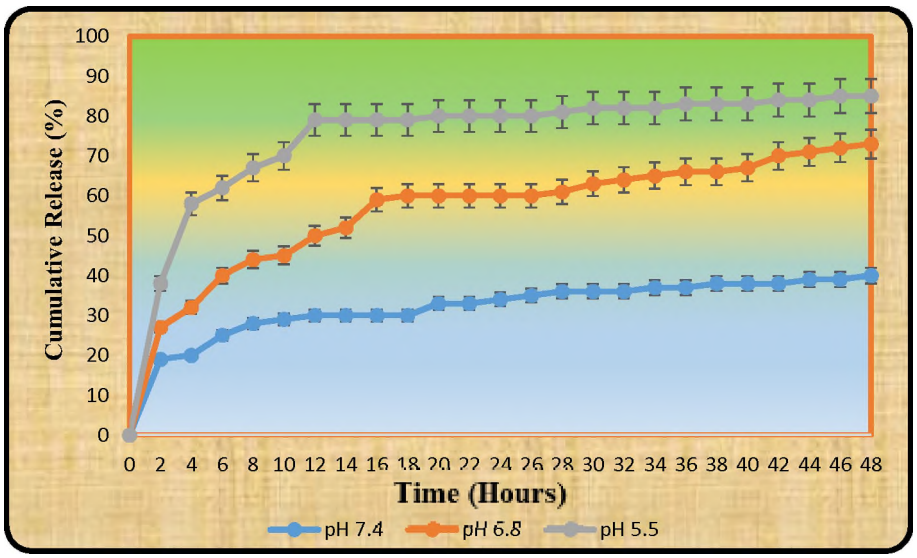
Similarly, several other studies have reported that lower toxicity for biologically synthesized silver nanoparticles than the chemically synthesized ones. Lin *et al.* (2010) reported that PEG coated on the surface of mesoporous silica nanoparticles did not resulted in apparent hemolysis of blood after 3hours of incubation. Similarly, the alcoholic extract of tulsi leaf mediated silver nanoparticles did not show any red blood cell lysis when compared with conventional drug (Khatoon *et al.*, 2015). In another study, the silver nanoparticles synthesized from aloevera exhibited low toxicity (Sadhasivam and Durairaj, 2014). Huang *et al.* (2016) evaluated silver nanoparticles towards blood compatibility and reported that the silver nanoparticles coated with polyvinyl pyrrolidine and citrate did not show any effect on hemolysis, platelet aggregation, coagulation process or complement activation at about 40µg/ml.

4.3.2 Drug Releasing Profiles of PEGylated AgNPs at Different pH

The toxicity study was followed by the drug release profiles of PEGylated AgNPs prepared from *Volkameria inermis* leaves from 0 hour upto 48 hours. The PEGylated AgNPs showed an obvious pH related release behavior. The profile showed that at pH 7.4 the release rate was slow and sustained with a release per cent of 40 ± 0.23 in 48 hours. At pH 6.8 the drug release was higher than that at pH 7.4 indicating the sensitivity of the drug towards tumor pH. The release per cent was found to be 73±0.29 in 48hours. At pH 5.5 the drug release was more rapid with approximately 85±0.39 within the same period (Figure 29).

Figure 29

Drug release profile of PEGylated AgNPs of *Volkameria inermis* leaves at different pH



At pH 7.4 the drug release was slow and sustained with a release per centage of 40±0.23 may be attributed to the hydrazide linkage of PEGylated AgNPs remained stable for a considerable period of time during circulation in blood and thereby eliminates a premature burst release. Such stability, for a prolonged period of time can reduce the side effects of the drug on normal cells. At pH 6.8 the drug release was higher than at pH 7.4, probably due to the slight protonation effect of the hydrazide linkage. However, at pH 5.5 a drastic release was obtained, since at lower pH degradation of hydrazide bond occurred

which enabled a drastic release of phytocomponents. Thus, this study showed the druggability of PEGylated AgNPs towards the tested pH conditions.

Thus, in all the pH conditions the drug release pattern was found to be steadily increased upto 16 hours, after which a steady plateau was obtained indicating the sustained release of the phytocompounds which improved the therapeutic efficacy without side effects. These results strongly supported the drugability of functionalized AgNPs at physiological pH, tumor pH and endosomal pH.

Mukherjee *et al.* (2014) reported that silver nanoparticles synthesized from the leaf of *Olax scandens* showed a high release of silver ions in acidic environment under *in vitro* conditions. Similarly, in another study the *in vitro* release of microanozle from microanozle AgNPs was found to be higher at acidic pH (Ganeshkumara and Poornachandraa, 2015).

The results obtained from phase III, revealed that PEGylated AgNPs synthesized from *Volkameria inermis* leaves extract was non-toxic and showed a steady release of phytocompounds, indicating PEGylated AgNPs as a very good biocompatible material in drug delivery system which can be applied in biomedical applications such as apoptotic and antitumor activity which were documented in Phase IV and V.

PHASE IV

4.4 APOPTOTIC EFFECT OF PEGYLATED AgNPs ON EAC CELL LINE

Apoptosis, referred as programmed cell death is characterized by the activation of early endogenous proteases, cell shrinkage and DNA fragmentation. In order, to reduce the tumor cell proliferation, the extent of apoptosis was found to be higher in cells associated with chemotherapy and radiotherapy (Orrenius *et al.*, 2011). The apoptotic activity of PEGylated AgNPs was evaluated against EAC cells by assessing cytotoxic effect, morphological changes by AO/EtBr dual staining, DNA fragmentation and cell cycle analysis.

4.4.1 Cytotoxic Effect of PEGylated AgNPs to EAC Cell Line by MTT Assay

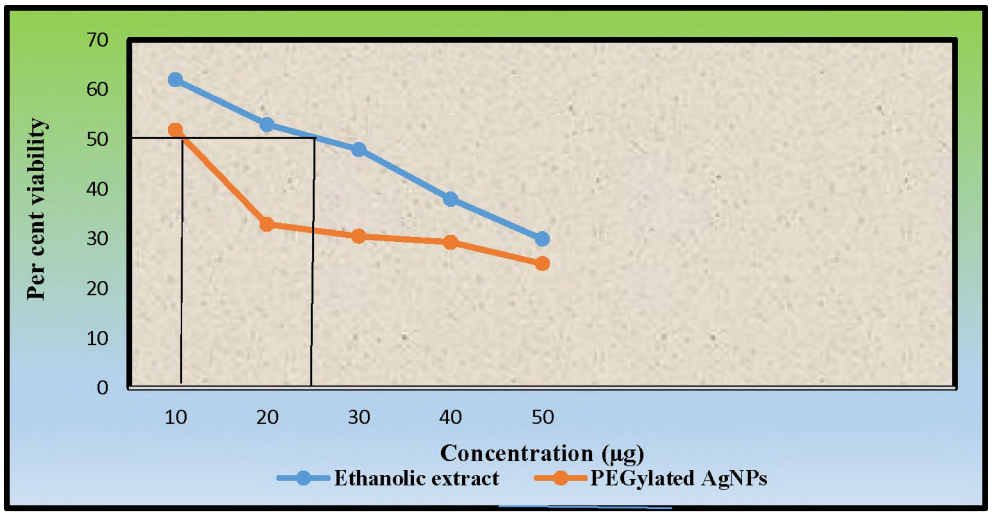
The *in vitro* cytotoxic study was used to determine the viability of cells. The cytotoxic potential of compounds can be analyzed and validated by MTT assay

(Sumantran, 2011) so, the antiproliferation assay like MTT was used in cytotoxicity studies. To determine the influence of PEGylated AgNPs on the viability of EAC cells. A dose dependent effect of PEGylated AgNPs was evaluated using a wide dose range of 10 µg to 50 µg/ml for both PEGylated AgNPs and the ethanolic leaves extract. The degree of viability observed for the PEGylated AgNPs and its ethanolic leaves extract at the same dose level was shown in Figure 30. Both the PEG functionalized AgNPs and etahnolic leaves extract showed a dose dependent decrease in the viability of EAC cells. The IC_{50} calculated for ethanolic leaves extract and PEGylated AgNPs was found to be 10 and 25 µg respectively.

These results showed that the PEGylated AgNPs and ethanolic leaves extract possess strong anticancer activity against EAC cells. The extent of viability was very much lower in PEGylated AgNPs compared to the ethanolic leaves extract. These observations clearly showed that the anticancer effect of ethanolic leaves extract can be increased two fold by administering them as PEGylated AgNPs.

Figure 30

Effect of PEGylated AgNPs on the viability of EAC cells (24 hours treatment)



Metallic nanoparticles such as silver, gold, iron, titanium and ruthenium has shown promising anticancer property in preclinical research (Ceresa *et al.*, 2014). Among the metallic nanoparticles silver and gold have drawn the attention of researchers because

of their unique physicochemical properties which can be used in biomedical applications such as bioimaging, biosensing, antimicrobial agents and cancer therapy (Luo *et al.*, 2014). Research has proved the successful usage of silver nanoparticles for cancer treatment. Silver nanoparticles not only interact with cells, but they have the tendency to mediate molecular processes and regulate cell functions (Wei *et al.*, 2015).

Several authors also reported the antiproliferative activity of AgNPs. A dose dependent decrease in the viability of human colon cancer HCT 15 cells were observed on treatment with silver nanoparticles synthesized from the ethanolic extract of rose petals (Manikandan *et al.*, 2015). The silver nanoparticles synthesized from the bark of *Moringa oleifera* possessed strong cytotoxic property against human cervical carcinoma cells (Vasanth *et al.*, 2014). The silver nanoparticles synthesized from the oak fruit showed a dose dependent toxic effect against human breast cancer cells (Heydari and Rashidipour, 2015).

The size and surface area of the silver nanoparticles play a major role towards cytotoxic nature. Silver coated with polyvinylpyrrolidone nanoparticles showed a strong cytotoxic effect due to its bigger surface area and high reactivity (Guo *et al.*, 2013). Another study conducted by Sathishkumar *et al.* (2014) also revealed that silver nanoparticles synthesized from *Dendrophthoe falcata* induced toxicity by the cellular uptake through clathrin dependent endocytosis and micropinocytosis.

The results of the present research work are in accordance with these studies. The size of the PEGylated silver nanoparticles was 25-35 nm, as shown in Phase II. This small size could be the causative factor for the high cytotoxic nature observed in EAC cells.

4.4.2 Effect of PEGylated AgNPs on the Morphological Changes in EAC Cell by AO/EtBr Dual Staining

As the anticancer activity of ethanolic leaves extract and PEGylated AgNPs was established, an attempt was made to perceive the type of cell death induced in the cancer cells, by AO/EtBr dual staining method. AO/EtBr is a differential staining method that can differentiate normal and apoptotic cells. The cancerous cells that were exposed to

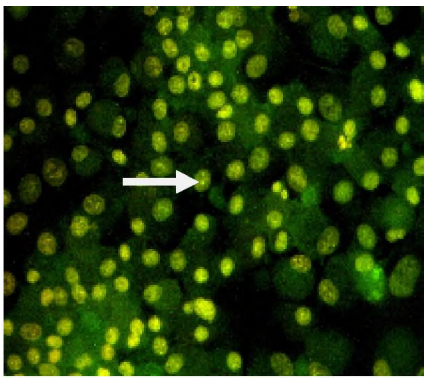
ethanolic leaves extract and PEGylated AgNPs were subjected to AO/EtBr staining and observed under fluorescent microscope. Apoptotic and necrotic cells stained orange while the normal cells stained green and were easily differentiated.

The microscopic study resulted that the type of cell death observed was predominantly apoptosis. The microscopic images are shown in Plate 7.

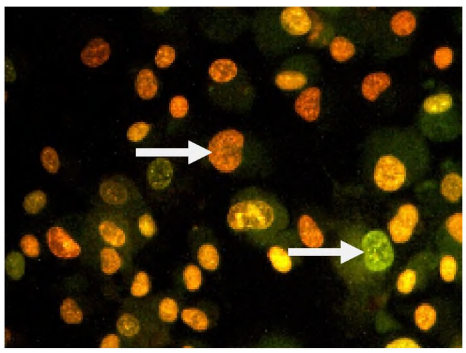
Plate 7

Apoptotic effect of ethanolic leaves extract and PEGylated AgNPs of *Volkameria inermis* on EAC cells by AO/EtBr staining

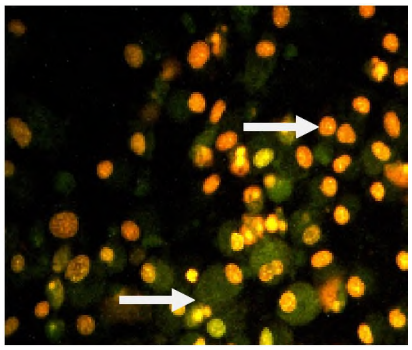
A) Control



B) Ethanolic leaves extract



C) PEGylated AgNPs



- **Typical morphological changes of EAC cells induced by IC_{50} of ethanolic leaves extract and PEGylated AgNPs**
- **The images were taken using fluorescence microscopy at 40X**
- **Arrows indicate the formation of apoptotic bodies, condensed nucleus and membrane blebbing as an evidence of PEGylated AgNPs induced apoptosis**
- **Living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange stained nuclei).**

In AO/EtBr dual staining, AO is a fluorescent dye which stains both live and dead cells, whereas EtBr stains only dead cells due to the loss of membrane integrity. Baharara *et al.* (2015) reported the morphological changes associated with MCF-7 breast cancer cells on treatment with silver nanoparticles synthesized from *Achillea biebersteinii* using AO/EtBr staining. Mata *et al.* (2015) also reported the induction of apoptosis using silver nanoparticles synthesized from *Pulmeria alba* against COLO 205 colon cancer cells by AO/EtBr staining method. The silver nanoparticles synthesized from *Ficus religiosa* showed strong apoptosis against Dalton lymphoma tumor cells studied by AO/EtBr staining method (Antony *et al.*, 2013). These research works provided further reliability to the observations obtained in the present study.

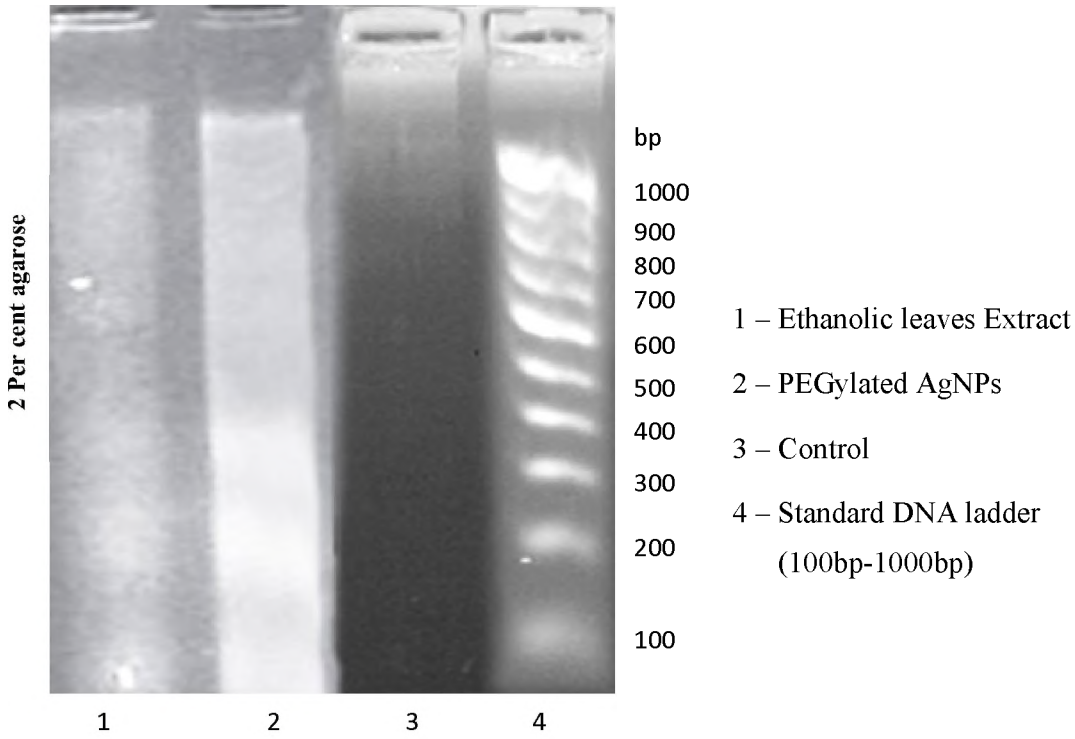
The *in vitro* study evidently occasioned that the ethanolic leaves extract of *Volkameria inermis*, possessed discernable anticancer activity which is mediated by the induction of apoptotic pathway. When the bioactive constituents of the extract were reduced to their nano form, the extent of apoptosis in the cancer cells increased significantly. Hence, this phase of the study clearly revealed the anticancer effect of the research plant *Volkameria inermis* and the activity could be improved significantly by converting the phytocomponents to PEGylated AgNPs.

4.4.3 Effect of PEGylated AgNPs on DNA Fragmentation in EAC Cell Line

Fragmentation of DNA occurs in programmed cell death and it was also noted in certain stages of necrosis. DNA damage was determined by agarose gel electrophoresis (Wang *et al.*, 2007).

Figure 31

DNA fragmentation in EAC cells treated with PEGylated AgNPs



In DNA fragmentation of EAC cells the DNA were depicted by its DNA ladder in Figure 31. Ethanolic leaves extract treated at the concentration of 25µg/ml significantly increased the DNA fragmentation of EAC cells (Lane 1). PEGylated AgNPs treated cells underwent more DNA fragmentation than cells treated with ethanolic leaves extract at the concentration of 10µg/ml (Lane 2). The DNA of control indicated no apoptosis (Lane 3).

Gandhiraj *et al.* (2015) reported significant anticancer activity of silver nanoparticles synthesized from the leaves of *Momordica charantia* against MCF – 7 breast cancer cell line. The cells were treated against plant extract and silver nanoparticle of *Momordica charantia* were subjected to DNA fragmentation assay. The silver nanoparticles showed higher percentage of DNA damage than the plant extract. Rosarin *et al.* (2012) reported that the PEGylated AgNPs synthesized from *Phyllanthus emblica* underwent more DNA fragment than cells treated with silver nanoparticles at the concentration of 30µg/ml.

Green synthesis of silver nanoparticles using *Ganoderma neo-japonicum Imazeki* showed a potential cytotoxic effect against breast cancer cells. The apoptotic effect of silver nanoparticles confirmed the DNA nuclear fragmentation (Gurunathan *et al.*, 2013).

The above results confirmed the arrest of cell proliferation by DNA fragmentation in the programmed cell death. Knowing the DNA fragmentation by PEG functionalized AgNPs an attempt was made to find out the stage at which the cell proliferation was arrested by cell cycle analysis.

4.4.4 Effect of PEGylated AgNPs on Cell Cycle Analysis by Flow Cytometry

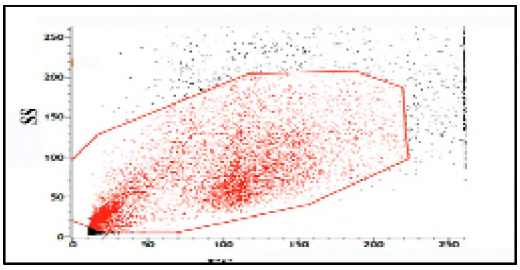
Having established the apoptotic type of cell death induced by PEG functionalized silver nanoparticles an effort was taken to determine the cellular events in the cell cycle by flow cytometry. Propidium iodide was used to record the cell cycle events by flow cytometry, after exposing the EAC cells for 24 hours to the ethanolic leaves extract and PEGylated AgNPs. The proportions of cells arrested in G_o / G_1 phase, S phase and G_2 / M phase of the cell cycle were quantified. The scattergrams and histograms obtained for the ethanolic leaves extract and PEGylated AgNPs were depicted in Figure 32 (i and ii).

Figure 32

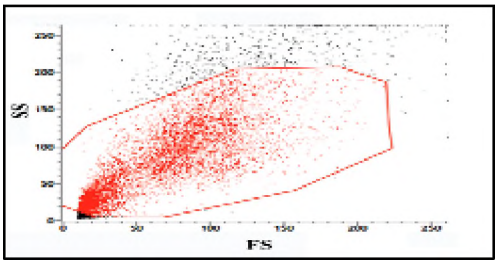
Effect of ethanolic leaves extract and PEGylated AgNPs on cell cycle events in EAC cells

i) Scattergrams

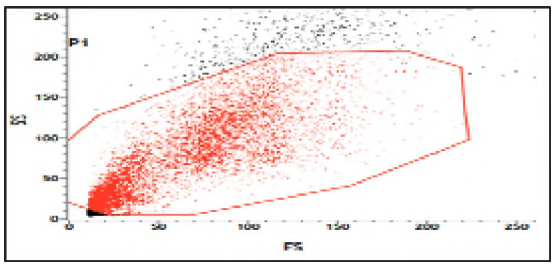
Control



Ethanolic extract



PEGylated AgNPs



ii) Histograms

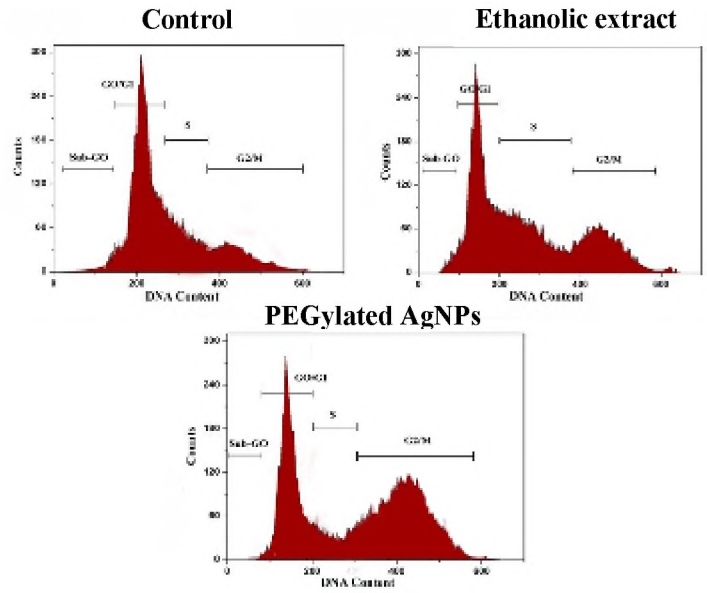


Table 3

Cell cycle analysis of ethanolic extract / PEGylated AgNPs in EAC cells

Phases of cell cycle	Per cent of cells		
	Control	Ethanolic extract	PEGylated AgNPs
Sub G_0	17.98	15.34	14.32
G_0 / G_1	48.91	40.32	37.39
S	27.40	19.40	15.90
G_2/ M	12.07	24.94	30.87

As shown in Figure 32 it was found that the ethanolic leaves extract and PEGylated AgNPs were able to inhibit the cell cycle at various phases. Untreated control, there was accumulation of cells in the G_0-G_1, S and G_2/M phase.The percentage of cells at the S-phase significantly decreased after treatment when compared to control. More cells commit to apoptosis when treated with PEGylated AgNPs. Moreover, the DNA duplication was arrested in respect to the treatment when compared to the control. The

result indicated the anti-proliferative effect of the particles could be predominantly derived from inducing cell cycle arrest mainly in DNA duplication phase.

In several literature reports, the arrest of cells in G_2 / M phase was recognized as high level of cytotoxicity. The silver nanoparticles synthesized from the ethanolic leaf extract of *Phytolacca decandra, Gelsemium sempervirens, Hydrastis canadensis* and *Thuja occidentalis* showed the highest peak in G_2/M phase, indicating the cytotoxic effect in human amelanotic melanoma (A375) cells (Das *et al.*, 2013). The silver nanoparticles of sodium citrate treated with HSC-3 oral cancer cells caused cell cycle arrest in G_2 / M phase (Austin *et al.*, 2011). Alshatwi *et al.* (2015) reported that the cell cycle analysis of human cervical cancer cells treated with the platinum nanoparticles synthesized from tea polyphenols showed G_2 / M phase arrest, due to DNA damage. Our results obtained are in agreement with these reports.

Differential staining by AO/EtBr staining recognized the cell death in the cancer cells as predominantly apoptosis. Cell cycle analysis by flow cytometry measurement lended support to this observation, that more cells were arrested at the G_2/M phase when exposed to the PEGylated AgNPs, implicating their commitment to the programmed cell death of apoptosis.The flow cytometry results also evidently supported the anticancer effect of *Volkameria inermis* leaves extract when PEGylated AgNPs were administered to EAC cells.

Thus, the results of Phase IV *in vitro* study exhibited strong anticancer activity of PEGylated AgNPs. The *in vitro* study was followed by *in vivo* (Phase V) study against EAC induced Swiss albino mice.

***In vivo* studies**

PHASE V

4.5 *In vivo* ANTIOXIDATIVE AND ANTITUMORIGENIC ACTIVITY OF PEGYLATED AgNPs IN EAC INDUCED SWISS ALBINO MICE

In the last phase of the research work *in vivo* study was carried out in Swiss albino mice. *In vivo* antioxidative potential of ethanolic leaves extract of *Volkameria inermis*

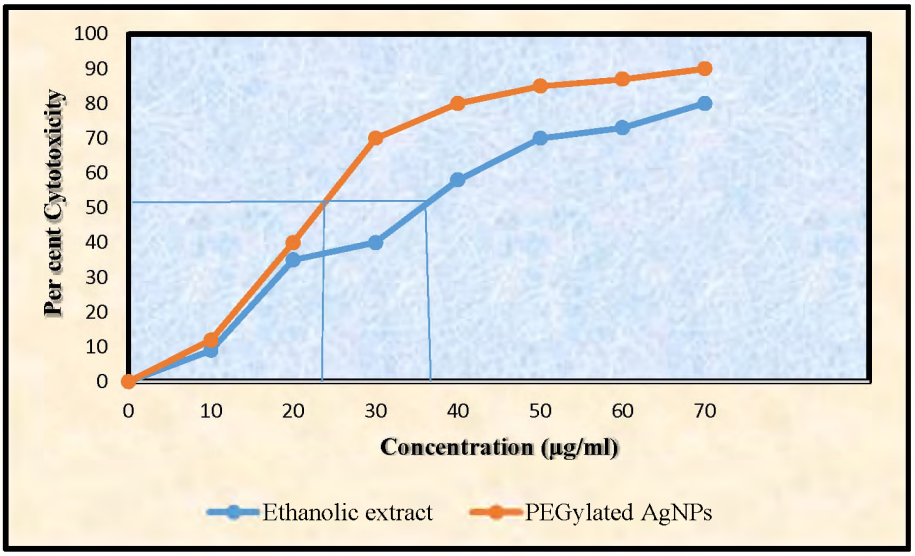
and PEGylated AgNPs in Swiss albino mice was evaluated by assessing the activities of enzymic and non enzymic antioxidants level and levels of lipid peroxides in comparison with the standard antioxidant silymarin for 15 days and 60 days treatment periods. The *in vivo* antitumorigenic effect of ethanolic extract of *Volkameria inermis* leaves and functionalized silver nanoparticles (PEGylated AgNPs) was assessed by evaluating the activities of liver marker enzymes namely aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) and increase in life span of EAC cells induced Swiss albino mice in the presence and absence of ethanolic extract of *Volkameria inermis* leaves and PEGylated AgNPs for 15 days and 60 days treatment periods. The histological status of the experimental mice was also evaluated.

4.5.1 Effect of PEGylated AgNPs on Antitumorigenic Activity to EAC Cells

In vivo antitumorigenic effect of PEGylated AgNPs was assessed by cytotoxic studies against intraperitoneally propagated EAC cells using trypan blue exclusion method. Figure 33 shows the dose dependent *in vitro* cytotoxic effect of PEGylated AgNPs to EAC cells.

Figure 33

Cytotoxic effect of PEGylated AgNPs to EAC cells by trypan blue method



Fifty per cent effective concentration (EC_{50}) was found to be 24µg/ml and 38 µg/ml for PEGylated AgNPs and ethanolic leaves extract respectively. This cytotoxic effect showed the antiproliferative role of ethanolic leaves extract and PEGylated AgNPs against EAC cells.

Taie *et al.* (2010) reported that the ethanolic and water extract of *Ocimum basilicum* possessed high cytotoxic and antioxidant activities against EAC Cells. The nanoparticles of *Temoxifen citrate* showed better cytotoxic activity against MCF-7 cancer cell line (Patel *et al.*, 2011). High cytotoxic and antioxidant activities was observed in the ethanolic extract of *Egyptian flora* against EAC cells (Enein *et al.*, 2012).

4.5.2 Effect of PEGylated AgNPs on the Mortality Rate of EAC Cells Induced Mice

The *in vivo* cytotoxic studies were carried out to follow the antitumor activity in terms of increase in life span of EAC cells bearing mice treated with ethanolic leaves extract and PEGylated AgNPs. The effect of ethanolic leaves extract and PEGylated AgNPs on the average life span of EAC cells bearing Swiss albino mice are shown in Table 4.

Table 4

Average life span of EAC cells induced mice treated with ethanolic leaves extract and PEGylated AgNPs of *Volkameria inermis*

Groups	EC_{50} in µg / ml/g.b.wt / 1 x 10^6 EAC cells	Average number of mice that survived after transplantation of EAC cells (days)						Average life span (days)
		10	20	30	40	50	60	
EAC	-	6/6	3/6	0/6	0/6	0/6	0/6	19
EAC+ Ethanolic leaves extract	38	6/6	6/6	6/6	5/6	5/6	4/6	46
EAC+ PEGylated AgNPs	24	6/6	6/6	6/6	6/6	6/6	6/6	60

The life span of EAC cells bearing mice was found to be 15-20 days with the average life span of 19 days. Coadministration of ethanolic leaves extract and PEGylated AgNPs inhibited the growth of EAC cells and increased the average life span to 46 and 60 days respectively and exhibited their antitumorigenic effect.

Intraperitoneal administration of ethanolic leaves extract and PEGylated AgNPs to tumor bearing mice, increased the life span of mice by detoxifying the tumor cells. In the present study, it was proved that the ethanolic leaves extract and PEGylated AgNPs have antioxidant activity which inturn increased the life span of EAC cells bearing mice and confirmed their antitumorigenic effect.

The findings were strongly supported by the previous research works. Roy *et al.* (2012) who reported that AgNPs showed promising safe anticancer drugs, since AgNPs have minimal impact on animal hematology. Administration of AgNPs of andrographolide and additional chitosan coating increased the anticancer efficacy of human breast cancer cells and EAC induced animal model. Liu *et al.* (2010) reported that trimethyl chitosan encapsulated Camptothecin (CPT-TMC) treated groups increased the life span to 42 days by actively antiproliferating B16-F10 cells. Rekha and Jayakar (2011) also reported that the ethanolic extract of *Butea monosperma* (Lam) Taub increased the lifespan of EAC cells bearing mice to 30 days. Zahan *et al.* (2011) reported that the crude extract of *Alangium salvifolim* significantly reduced the tumor volume, weight, viable cell count and increased the life span of EAC cells bearing mice. The methanolic leaf extract of *Leea indica* heightened the life span of EAC induced Swiss albino mice to 69.33 per cent (Raihan *et al.*, 2012). Jayaseelan *et al.* (2012) reported that the methanolic root extract of *Desmodium triangulare* increased the life span of EAC and DLA to 50 days and 64 days respectively. Nahar *et al.* (2012) reported that the flowers of *Alangium salvifolim* increased the life span of EAC cell bearing mice to 40 days.

The study concluded that the PEGylated AgNPs increased the life span of EAC cells bearing mice and proved the antitumorigenic effect by its antiproliferative role.

4.5.3 Effect of PEGylated AgNPs on the Activities of Liver Marker Enzymes in Serum of EAC Challenged Swiss albino Mice

In order to find out the normal functioning of liver, the liver marker enzymes namely AST, ALT and ALP were assessed in the serum of all the control and experimental groups and are shown in Tables 5, 6 and 7.

4.5.3.1 Effect on aspartate transaminase (AST)

The activity of AST was found to be significantly reduced in standard antioxidant silymarin when compared to the control paraffin oil in both 15 days and 60 days treatment periods. Administration of ethanolic leaves extract and PEGylated AgNPs exhibited significant decrease in the activity of AST when compared to the DMSO control group (Figure 34).

The activity of AST was found to be increased in EAC cells induced mice in 15 days treatment period when compared to all the control and other experimental groups. Administration of ethanolic leaves extract and PEGylated AgNPs to EAC cells induced mice showed significant decrease in AST activity in 60 days when compared to 15 days treatment period.

Table 5

Activity of AST in the serum of control and experimental Swiss albino mice

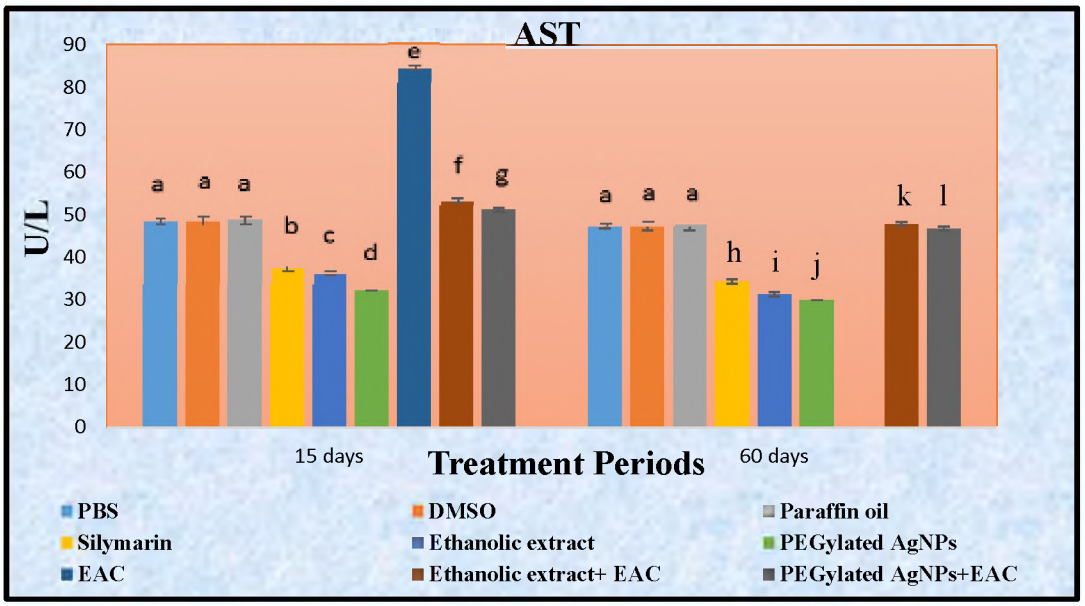
Groups	AST (U/L) [a]	
	15 Days	60Days
PBS	48.23 ± 0.553	47.30 ± 0.538
DMSO	48.32 ± 1.106	47.22 ± 1.048
Paraffin oil	48.46 ± 1.091	47.41 ± 1.079
Silymarin	37.22 ± 0.533	34.33 ± 0.538
Ethanolic extract	36.06 ± 0.524	31.3 ± 0.525
PEGylated AgNPs	35.23 ± 0.521	30.97 ± 0.507
EAC	84.24 ± 0.516	-
Ethanolic extract +EAC	53.05 ± 0.535	47.82 ± 0.519
PEGylated AgNPs +EAC	51.19 ± 0.035	46.76 ± 0.566
One way ANOVA with EAC (P< 0.05)	0.637	-
One way ANOVA without EAC (P< 0.05)	0.635	0.706
Two way ANOVA without EAC (P< 0.05)	0.635	

The values are mean ± SD of six animals

(U/L) [a] micromole of pyruvate formed/minute/mg of protein

Figure 34

Effect of ethanolic leaves extract and PEGylated AgNPs on the serum of AST in control and EAC induced Swiss albino mice



The values are mean ± SD of six animals

Means which are followed by different superscripts differ significantly at P < 0.05

Means which are followed by same superscripts do not differ significantly at P<0.05

Research findings of Santhi and Annapoorani (2009) showed that administration of leaf protein from *Terminalia catapapa* decreased the level of AST, ALT and ALP in EAC induced Swiss albino mice. A significant decrease in the level of AST and ALT were observed by the administration of silymarin to CCl_4 induced hepatotoxicity in mice (Ravikumar *et al.*, 2010). A significant decrease in the activities of AST, ALT and ALP were noticed in CCl_4 induced rats in a dose dependent manner by the administration of *Zizyphus spina-christi* fruit powder (Heba *et al.*, 2011). Nalini *et al.* (2011) observed a significant decrease in the activity of AST in the serum of EAC induced mice administered with *Cissus quadrangularis* extract.

4.5.3.2 Efect on alanine transaminase (ALT)

The standard antioxidant silymarin, showed a significant decrease in ALT activity when compared to the control paraffin oil at 15 days and 60 days. Administration of

ethanolic leaves extract and PEGylated AgNPs showed significant decrease in ALT activity when compared to DMSO control in both 15 days and 60 days treatment periods (Figure 35).

The activity of ALT was found to be increased in EAC cells induced mice in 15 days treatment period when compared to all the control and other experimental groups. Coadministration of ethanolic leaves extract and PEGylated AgNPs significantly reduced the activity of ALT in 15 days and 60 days treatment periods.

Table 6

Activity of ALT in the serum of control and experimental Swiss albino mice

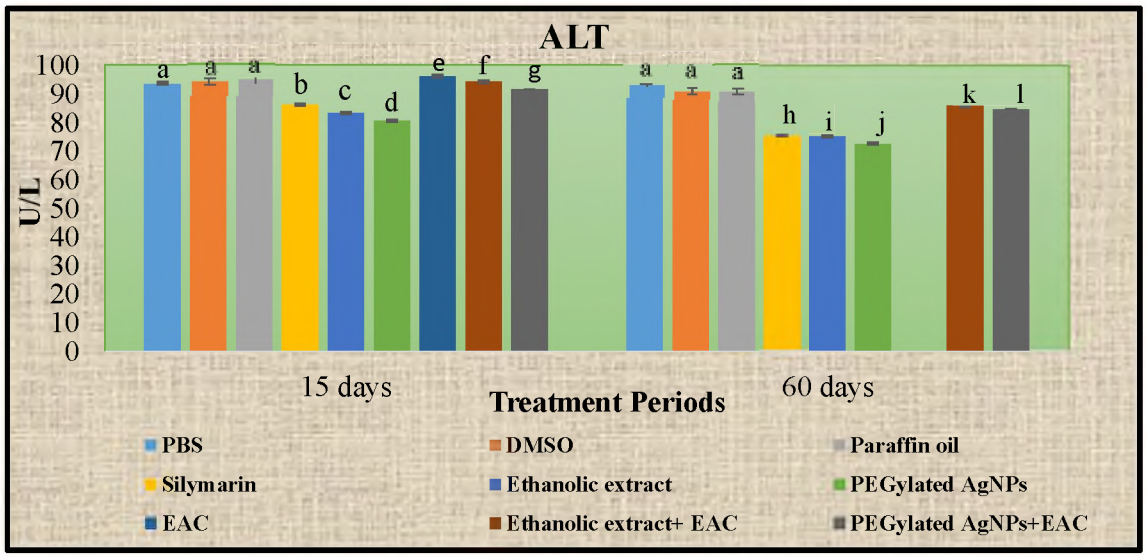
Groups	ALT (U/L) [b]	
	15 Days	60Days
PBS	93.53 ± 0.526	92.43 ± 0.551
DMSO	92.49 ± 1.017	90.40 ± 1.033
Paraffin oil	94.34 ± 1.099	90.49 ± 1.043
Silymarin	86.22 ± 0.533	75.52 ± 0.538
Ethanolic extract	83.24 ± 0.531	75.14 ± 0.535
PEGylated AgNPs	82.56 ± 0.036	74.62 ± 0.016
EAC	96.07 ± 0.544	-
Ethanolic extract +EAC	94.06 ± 0.551	85.83 ± 0.513
PEGylated AgNPs +EAC	93.84 ± 0.517	84.76 ± 0.553
One way ANOVA with EAC ($P< 0.05$)	0.745	-
One way ANOVA without EAC ($P< 0.05$)	0.742	0.672
Two way ANOVA without EAC ($P< 0.05$)	0.742	

The values are mean ± SD of six animals

(U/L) [b] micromole of pyruvate formed/minute/mg of protein

Figure 35

Effect of ethanolic leaves extract and PEGylated AgNPs on the serum of ALT in control and EAC induced Swiss albino mice



The values are mean ± SD of six animals

Means which are followed by different superscripts differ significantly at P < 0.05
Means which are followed by same superscripts do not differ significantly at P<0.05

A significant decrease in the activity of liver marker enzymes namely AST, ALT and ALP were observed by the administration of ethanolic extract of *Clausena dentata* to paracetamol induced hepoatotoxicity in rats (Rajesh *et al.*, 2009). The alcoholic extract of *Cassia alata* leaves normalized the levels of AST, ALT and ALP in a dose dependent manner (Neharkar and Gaikwad 2011).

4.5.3.3 Effect on alkaline phosphatase (ALP)

The standard antioxidant silymarin, showed a significant decrease in ALP activity when compared to the control paraffin oil at 15 days and 60 days. Ethanolic leaves extract and PEGylated AgNPs administration showed significant decrease in ALT activity when compared to DMSO group in both 15 days and 60 days treatment periods (Figure 36).

The activity of ALP was found to be increased in EAC cells induced mice in 15 days treatment period when compared to all the control and other experimental groups. Coadministration of ethanolic leaves extract and PEGylated AgNPs significantly reduced the activity of ALP in 15 and 60 days treatment periods.

Table 7

Activity of ALP in the serum of control and experimental Swiss albino mice

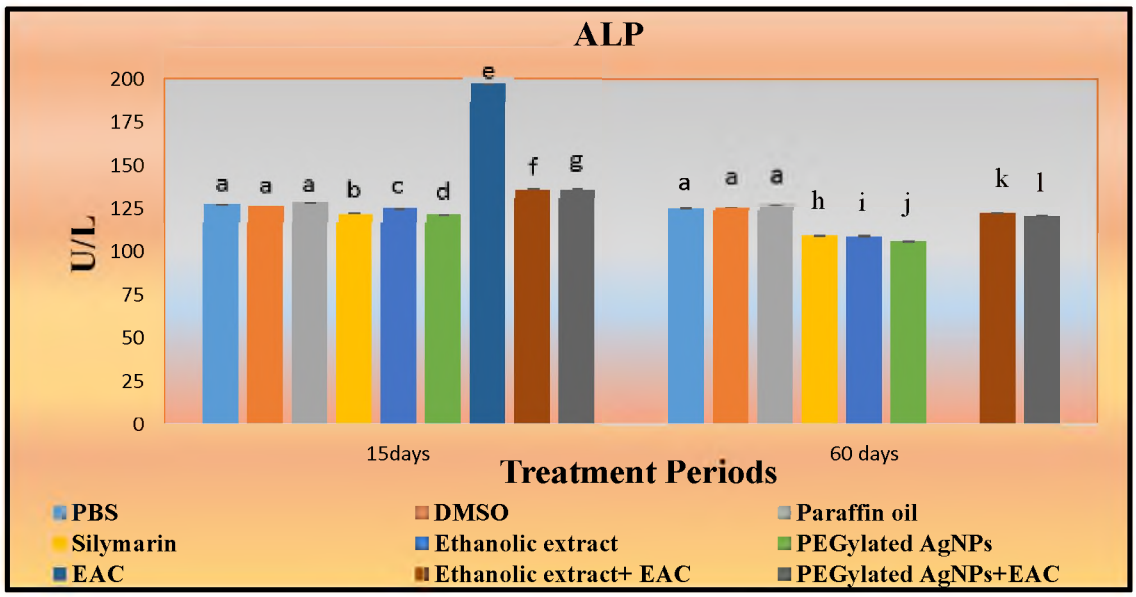
Groups	ALP (U/L) [c]	
	15 Days	60Days
PBS	126.86 ± 0.016	124.84 ± 0.024
DMSO	125.82 ± 0.037	124.74 ± 0.020
Paraffin oil	127.84 ± 0.042	126.64 ± 0.076
Silymarin	121.56 ± 0.033	109.04 ± 0.016
Ethanolic extract	124.56 ± 0.025	108.65 ± 0.016
PEGylated AgNPs	123.57 ± 0.032	107.64 ± 0.017
EAC	196.57 ± 0.014	-
Ethanolic extract +EAC	135.56 ± 0.023	122.15 ± 0.216
PEGylated AgNPs +EAC	135.18 ± 0.008	120.26 ± 0.021
One way ANOVA with EAC (P< 0.05)	0.031	-
One way ANOVA without EAC (P< 0.05)	0.030	0.753
Two way ANOVA without EAC (P< 0.05)	0.030	

The values are mean ± SD of six animals

(U/L) [c] micromole of phenol liberated/minute/mg of protein

Figure 36

Effect of ethanolic leaves extract and PEGylated AgNPs on the serum of ALP in control and EAC induced Swiss albino mice



The values are mean ± SD of six animals

Means which are followed by different superscripts differ significantly at P < 0.05

Means which are followed by same superscripts do not differ significantly at P<0.05

Increase in the level of AST, ALT and ALP were detected in the serum of CCl_4 induced rats which indicated liver dysfunction and these levels were brought down in rats on administration of ethanolic extract of *Vitex trifolia* flowers (Anandan *et al.,* 2009). A significant decrease in the activities of AST, ALT and ALP was noticed in rats administered with the ethanolic extract of *Spirulina lonar* in paracetamol induced liver damage (Kuriakose and Kurup, 2010). The altered levels of biochemical markers were brought back to normal range on administration of methanolic fruit extract of *P.domestica* to mice (Manoj Soni *et al.*, 2011)

The EAC cells induced mice showed an increase in the liver function test enzymes. This effect was backslid towards the normal level on administration of ethanolic leaves extract and PEGylated AgNPs. Of which the PEGylated AgNPs showed more significant effect than that of the ethanolic leaf extract.

The liver not only perform physiological functions but also play an important role in detoxifying harmful chemicals and metabolizes drugs (Guerra *et al.*, 2016). Due to hepatocellular damage in EAC induced mice, an elevation in the level of liver marker enzymes in serum occurs. This might be due to the leakage of cytosolic enzymes into the circulatory system. Hepatocellular damage leads to liver dysfunction, disturbance in the biosynthesis of liver marker enzymes, with alteration in the permeability of liver membrane. Administration of ethanolic leaves extract and PEGylated AgNPs altered the status of liver marker enzymes and brought back to normal range. Thus, it maintains the hepatocellular membrane integrity. The hepatoprotective property of ethanolic leaves extract and PEGylated AgNPs was found to be on par with silymarin the standard antioxidant and indicated their antioxidant potential in maintaining the normal functioning of liver. Moreover, the antioxidant role of PEGylated AgNPs was found to be significant than the ethanolic leaves extract.

The extent of liver damage in EAC induced mice was determined by assessing the level of liver marker enzymes such as AST, ALT and ALP (Dobbs *et al.*, 2003). Elevation in the level of AST, ALT and ALP occurs in EAC cells bearing mice due to the alteration of membrane integrity of liver. Ramprasath *et al.* (2007) reported that the extent of liver damage was predicted by the levels of liver marker enzymes in the serum of carcinoma mice. These enzymes AST, ALT and ALP were associated with direct

conversion of amino acids to ketoacids. Increase in the level of ALT was associated with the increase in the level of ALP (Mohan Rao *et al.*, 1989). Treatment with ethanolic leaves extract and PEGylated AgNPs brought the elevated activities of these enzymes to normal range.

4.5.4 Effect of PEGylated AgNPs on the Activities of Enzymic Antioxidants

Free radicals are constantly produced in living cells as a part of normal metabolism. Excess production of free radicals are toxic to humans causing various vulnerable diseases. To overcome this defect the excess free radicals are counteracted by antioxidative defense enzymes namely catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). Liver plays an important role in drug metabolism by eliminating the drug toxicity. Hence, the activities of CAT, SOD and GPx was assessed in the liver of Swiss albino mice by administering with and without ethanolic leaves extract and PEGylated AgNPs against EAC cells induced mice and were compared with the standard antioxidant treated mice for the treatment periods 15 days and 60 days.

4.5.4.1 Effect on catalase (CAT)

The activity of catalase was found to be increased in mice treated with silymarin, ethanolic leaves extract and PEGylated AgNPs in all the treatment periods when compared with their corresponding control. Administration of ethanolic leaves extract and PEGylated AgNPs showed more significant increase in catalase activity than that of silymarin. The EAC cells induced mice showed significant decrease in CAT activity on 15 days treatment period when compared with the control and all other treatment groups. The catalase activity was found to be significantly increased in ethanolic leaves extract and PEGylated AgNPs in both the treatment periods. Coadministration of ethanolic leaves extract and PEGylated AgNPs to EAC cells induced mice on all treatment periods significantly increased catalase activity in 60 days treatment period when compared to 15 days treatment period (Figure 37).

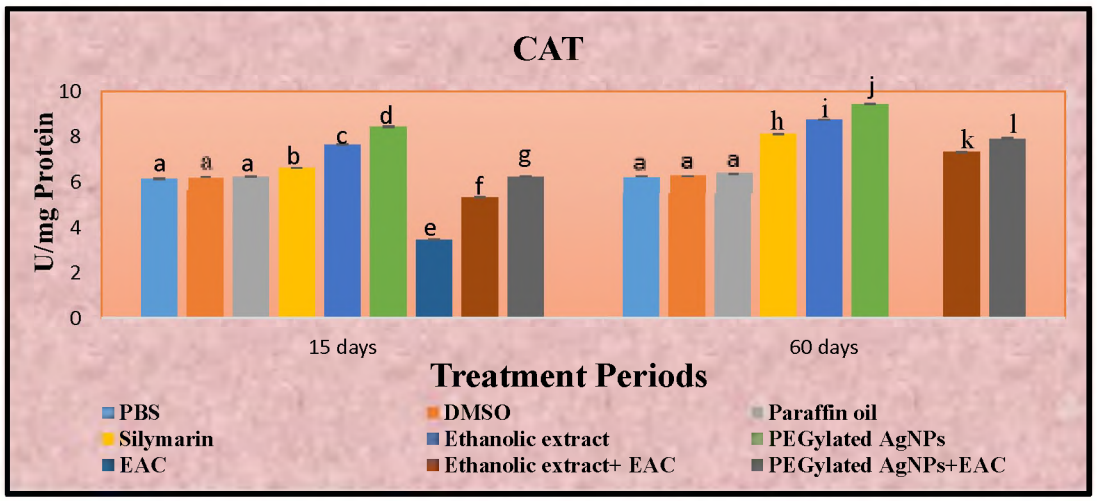
Table 8
Activity of catalase in hepatic tissues of control and experimental Swiss albino mice

Groups	Catalase (U^a/mg protein)	
	15 Days	60Days
PBS	6.15 ± 0.296	6.23 ± 0.121
DMSO	6.16 ± 0.074	6.27 ± 0.063
Paraffin oil	6.15 ± 0.008	6.32 ± 0.040
Silymarin	6.63 ± 0.018	8.12 ± 0.014
Ethanolic extract	7.66 ± 0.038	8.76 ± 0.015
PEGylated AgNPs	8.44 ± 0.167	9.44 ± 0.018
EAC	3.46 ± 0.026	-
Ethanolic extract + EAC	5.34 ± 0.008	7.33 ± 0.015
PEGylated AgNPs +EAC	6.24 ± 0.198	7.92 ± 0.007
One way ANOVA with EAC(P<0.05)	0.174	-
One way ANOVA without EAC (P< 0.05)	0.173	0.046
Two way ANOVA without EAC (P< 0.05)	0.173	

The values are mean ± SD of six animals
U^a- Amount of enzyme required to decrease the absorbance by 0.05 units at 240nm.

Figure 37

Effect of ethanolic leaves extract and PEGylated AgNPs on the activities of CAT in the liver of control and EAC induced Swiss albino mice



The values are means ± SD of six animals

Means which are followed by different superscripts differ significantly at P < 0.05

Means which are followed by same superscripts do not differ significantly at P<0.05

Significant elevation in the activity of CAT on administration of ethanolic leaves extract and PEGylated AgNPs might be due to the potent antioxidant activity of their active phytoconstituents. The antioxidant activity effectively scavenges the free radicals generated due to tumor burden.

Catalase is an enzymatic antioxidant found in all living organisms exposed to oxygen such as bacteria, plants and animals. The highest activity of catalase in animals was found in the red cells and liver. It catalyses the decomposition of hydrogen peroxide to oxygen and water and protects the tissue from hydroxyl radicals (Gaetani *et al.*, 2017). The decrease in the activity of catalase in EAC cells bearing mice was due to the increased production of hydrogen peroxide. The administration of ethanolic leaves extract and PEGylated AgNPs significantly enhanced CAT activity and indicated their antioxidative role. However, the PEGylated AgNPs showed a marked increase in the activity of CAT compared to the ethanolic leaves extract. The catalase activity was decreased in hepatocellular carcinoma and it was reverted by ursolic acid administration (Gayathri *et al.*, 2010).

The significant decrease in the antioxidant activity of CAT, SOD and GPx in EAC cells bearing mice was restored to normal range on administration of the ethanolic leaf extract of *Aloe vera* (Naveena *et al.*, 2011). Prabha and Annapoorani (2011) reported that a significant increase in the activity of CAT in CCl_4 induced mice on administration with *Cynodon dactylon* leaf protein.

4.5.4.2 Effect on superoxide dismutase (SOD)

The activity of SOD was found to be significantly increased in mice administered with silymarin, ethanolic leaves extract and PEGylated AgNPs when compared to their control at 15 days and 60 days of treatment. Mice administered with PEGylated AgNPs showed more significant increase in SOD activity than that found in silymarin treatment. On 15 days treatment period the EAC cells induced mice showed a significant decrease in SOD activity when compared to other groups. Administration of ethanolic leaves extract and PEGylated AgNPs individually and to EAC cells induced mice showed a significant increase in the activity of SOD in 60 days treatment period when compared to 15 days treatment period (Figure 38).

Table 9

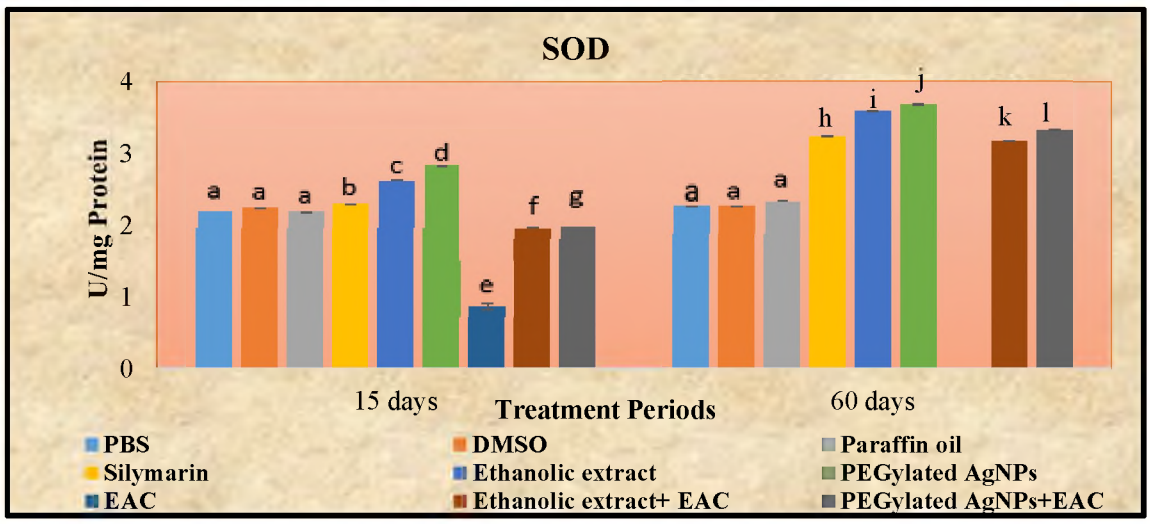
Activity of SOD in hepatic tissues of control and experimental Swiss albino mice

Groups	Superoxide dismutase (U^b /mg protein)	
	15 Days	60Days
PBS	2.17 ± 0.057	2.24 ± 0.524
DMSO	2.22± 0.043	2.24 ± 0.078
Paraffin oil	2.16 ± 0.283	2.32 ± 0.226
Silymarin	2.27 ± 0.037	3.23 ± 0.068
Ethanolic extract	2.60 ± 0.123	3.58 ± 0.112
PEGylated AgNPs	2.80 ± 0.043	3.67 ± 0.044
EAC	0.84 ± 0.012	-
Ethanolic extract+ EAC	1.94 ± 0.021	3.16 ± 0.036
PEGylated AgNPs +EAC	1.96 ± 0.076	3.32 ± 0.021
One way ANOVA with EAC(P<0.05)	0.077	-
One way ANOVA without EAC (P< 0.05)	0.076	0.095
Two way ANOVA without EAC (P< 0.05)	0.076	

The values are mean ± SD of six animals
U^b- Amount of enzyme that gives 50 per cent inhibition of the extent of NBT reduction/min.

Figure 38

Effect of ethanolic leaves extract and PEGylated AgNPs on the activities of SOD in the liver of control and EAC induced Swiss albino mice



The values are means ± SD of six animals
Means which are followed by different superscripts differ significantly at P < 0.05

Means which are followed by same superscripts do not differ significantly at P<0.05

A significant decrease in SOD activity was observed in EAC cells induced mice when compared to all the control and experimental mice in both the treatment periods. Administration of ethanolic leaves extract and PEGylated AgNPs individually and in coadministration with EAC cells induced mice showed significant increase in the activity of SOD when compared to 15 days treatment. Superoxide dismutase is an enzymatic antioxidant that converts superoxide radical into molecular oxygen and hydrogen peroxide. It plays an important role in defense mechanism in all living cells exposed to oxygen (Karavolos *et al.*, 2003). A decrease in SOD activity in EAC cells was due to the loss of Mn-SOD activity and the loss of mitochondria in liver (Sun *et al.*, 1989). *Momordia charantia* fruit hexane extract significantly increased the SOD activity in CCl_4 induced rats (Semiz and Sen, 2007). The EAC cells induced mice showed a significant decrease in SOD activity (Muthuraman *et al.*, 2008). The administration of methanolic fruit extract of *Garcinia gummi gutta* increased the SOD activity in paracetamol intoxicated rats (Tamizh Selvam *et al.*, 2011). The ethanolic extract of *Curcuma longa* and *Curcumin* rhizome increased the SOD activity in aflotoxin B1 induced mice (Veena *et al.*, 2011).

4.5.4.3 Effect on glutathione peroxidase (GPx)

Mice administered with ethanolic leaves extract, PEGylated AgNPs and silymarin showed a significant increase in GPx activity in all treatment periods when compared to the control (Figure 39). Ethanolic leaves extract and PEGylated AgNPs showed more GPx activity in 15 days and 60 days treatment periods than that of the standard antioxidant silymarin.

The EAC cells induced mice showed a significant decrease in the activity of GPx in 15 days treatment period when compared to the control and other treatment groups. The activity of GPx in EAC cells induced mice administerd with PEGylated AgNPs and ethanolic leaves extract exhibited a significant increase in 60 days treatment when compared to 15 days treatment period.

Table 10

Activity of GPx in hepatic tissues of control and experimental Swiss albino mice

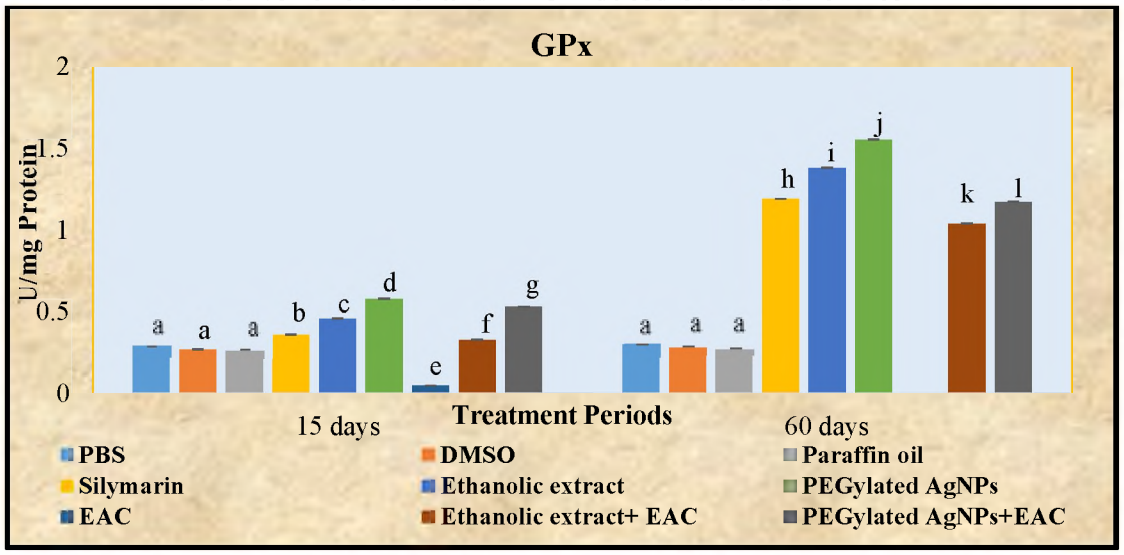
Groups	Glutathione peroxidase (U^c /mg protein)	
	15 Days	60Days
PBS	0.29 ± 0.005	0.30 ±0.013
DMSO	0.27 ± 0.008	0.28 ±0.008
Paraffin oil	0.26 ± 0.026	0.27± 0.034
Silymarin	0.36 ± 0.021	1.19 ±0.008
Ethanolic extract	0.46 ± 0.019	1.38 ±0.011
PEGylated AgNPs	0.58 ± 0.005	1.55 ±0.014
EAC	0.05 ± 0.008	-
Ethanolic extract+ EAC	0.33 ± 0.026	1.04±0.012
PEGylated AgNPs +EAC	0.53 ± 0.016	1.17±0.015
One way ANOVA with EAC(P<0.05)	0.018	-
One way ANOVA without EAC (P< 0.05)	0.019	0.023
Two way ANOVA without EAC (P< 0.05)	0.017	

The values are mean ± SD of six animals

U^c- Nanomoles of GSH oxidized / min / mg protein.

Figure 39

Effect of ethanolic leaves extract and PEG ylated AgNPs on the activity of GPx in the liver of control and EAC induced Swiss albino mice



The values are mean ± SD of six animals

Means which are followed by different superscripts differ significantly at P < 0.05

Means which are followed by same superscripts do not differ significantly at P<0.05

Low activity of GPx in EAC cells induced mice was due to more utilization of GPx in combating the increase in the generation of free radicals caused by the disturbance of cellular membrane rigidity in tumor burden. The activity was significantly increased by the administration of PEGylated AgNPs in EAC induced mice which showed the importance of nano in destroying oxygen free radicals generated by EAC cells.

The ethanolic extract of *Zingiber officinale* significantly increased the activity of SOD, CAT, GSH, GP_X and GST in aceclofenac induced rats (Pal *et al.*, 2010). Anbarasu *et al.* (2011) reported that administration of silymarin and the methanolic extract of *Pisonia aculeate* leaves increased the activities of antioxidant enzymes.

An effective defense mechanism was offered by enzymatic antioxidants such as CAT, SOD and GP_X which protects the cellular constituents from oxidative damage by neutralizing the free radicals. Administration of ethanolic leaves extract and PEGylated AgNPs significantly increased the level of CAT, SOD and GP_X which indicated the greater level of endogenous antioxidant production which enhanced free radical scavenging activity. A significant decrease in the activities of CAT, SOD and GP_X was found in EAC cells induced mice which revealed the excess production of free radicals and activation of lipid peroxidation system resulting in tissue damage. These free radicals are effectively scavenged by the antioxidative role of ethanolic leaves extract and PEGylated AgNPs. Moreover, the PEGylated AgNPs showed a greater antioxidative role when compared with the ethanolic leaves extract.

4.5.5 Effect of PEGylated AgNPs on the Levels of Non Enzymic Antioxidants

Free radicals are produced during normal aerobic metabolism. These free radicals are neutralized *in vivo* by a team of antioxidants. A relative deficiency in antioxidative defense causes oxidative stress which is associated with diseases like cancer (Grigorescu *et al.*, 2015). Hence, in the present research work the level of non enzymic antioxidants such as Vitamin A, E and reduced glutathione were assessed in the liver of control and experimental mice.

Table 11

Levels of Vitamin A in hepatic tissues of control and experimental Swiss albino mice

Groups	Vitamin A (mg/g tissue)	
	15 Days	60Days
PBS	0.69 ± 0.005	0.75 ± 0.13
DMSO	0.64 ± 0.008	0.63 ± 0.005
Paraffin oil	0.66 ± 0.026	0.67 ± 0.070
Silymarin	0.86 ± 0.021	2.02 ± 0.013
Ethanolic extract	0.94 ± 0.014	2.34 ± 0.021
PEGylated AgNPs	1.96 ± 0.014	2.36 ± 0.029
EAC	0.65 ± 0.008	-
Ethanolic extract +EAC	0.83 ± 0.016	1.04 ± 0.020
PEGylated AgNPs +EAC	0.95 ± 0.037	2.02 ± 0.017
One way ANOVA with EAC (P< 0.05)	0.018	-
One way ANOVA without EAC (P< 0.05)	0.017	0.148
Two way ANOVA without EAC (P< 0.05)	0.017	

The values are mean ± SD of six animals

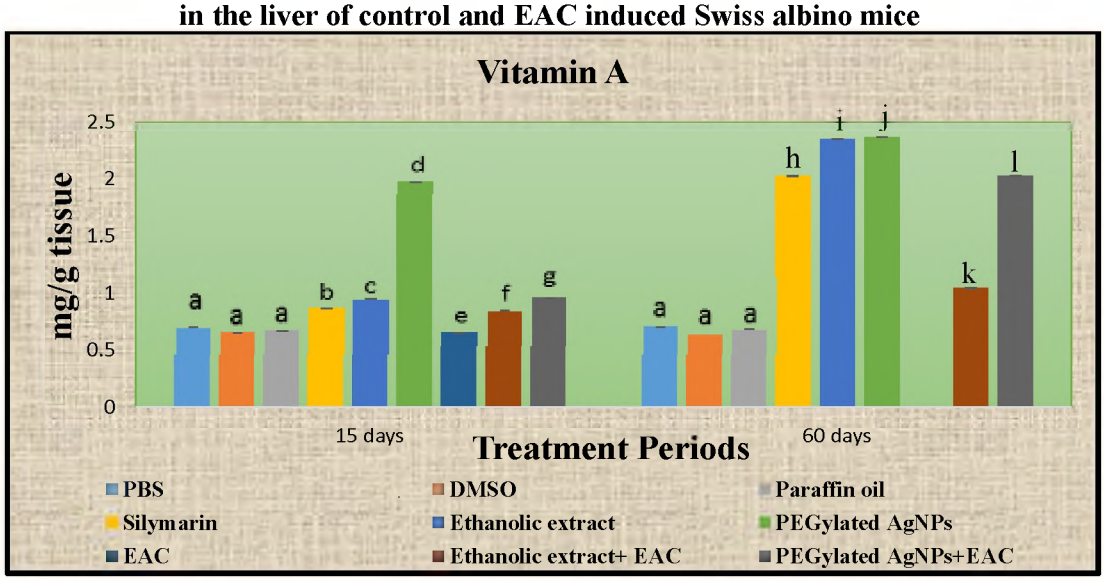
4.5.2.1 Effect on Vitamin A

Silymarin, the standard antioxidant treated group showed a significant increase in the level of Vitamin A during 15 days and 60 days of study period when compared to mice treated with paraffin oil. A significant increase in Vitamin A was found in mice treated with ethanolic leaves extract and PEGylated AgNPs. Compared to the ethanolic leaves extract, the PEGylated AgNPs exposed an increase in the level of Vitamin A (Figure 40).

The EAC cells induced mice showed a decrease in the level of Vitamin A on 15 days treatment period when compared to the control. On administration of ethanolic leaves extract and PEGylated AgNPs to mice induced with EAC showed an increase in the level of VitaminA.

Figure 40

Effect of ethanolic leaves extract and PEGylated AgNPs on the levels of Vitamin A in the liver of control and EAC induced Swiss albino mice



The values are mean ± SD of six animals

Means which are followed by different superscripts differ significantly at P < 0.05

Means which are followed by same superscripts do not differ significantly at P<0.05

A decrease in the level of Vitamin A was noted in EAC cells induced mice which was due to the liberation of lipid peroxide and tumor burden. Vitamin A is a natural antioxidant which neutralizes the free radicals and exerts its antioxidant effect (Wang *et al.*, 2008). The leaf protein of *Cynodon dactylon* increases the level of Vitamin A in EAC induced mice (Santhi and Annapoorani, 2010). The levels of non enzymic antioxidants such as Vitamin A, E, C and GSH were found to be increased in rat treated with *S. sahendica* extract (Esmaeili *et al.*, 2009).

4.5.2.2 Effect on Vitamin E

A significant increase in the level of Vitamin E was found in mice administered with PEGylated AgNPs when compared to control mice in 15 days and 60 days of treatment periods (Table 12 and Figure 41). Compared to paraffin oil control the silymarin treated mice showed significant increase in the level of Vitamin E in all the treatment periods. On 15 days and 60 days treatment periods the level of Vitamin E was increased significantly in PEGylated AgNPs than in ethanolic leaves extract. The level of Vitamin E was found to be significantly decreased in EAC cells induced mice in 15 days

treatment period. But the coadministration of PEGylated AgNPs and ethanolic leaves extract to EAC induced animals showed significant increase in the level of Vitamin E.

Table 12

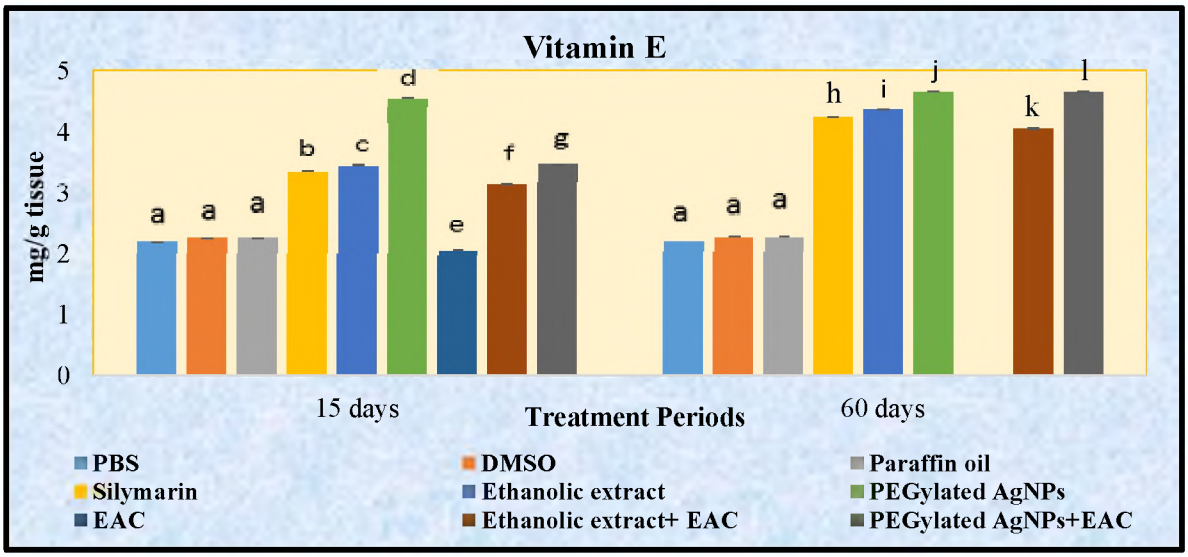
Levels of Vitamin E in hepatic tissues of control and experimental Swiss albino mice

Groups	Vitamin E (mg/g tissue)	
	15 Days	60Days
PBS	2.17 ± 0.016	2.18 ± 0.007
DMSO	2.24 ± 0.020	2.25 ± 0.007
Paraffin oil	2.24 ± 0.010	2.25 ± 0.040
Silymarin	3.33 ± 0.015	4.23 ± 0.018
Ethanolic extract	3.43 ± 0.012	4.36 ± 0.014
PEGylated AgNPs	4.53 ± 0.017	4.65 ± 0.010
EAC	2.04 ± 0.014	-
Ethanolic extract +EAC	3.13 ± 0.019	4.04 ± 0.020
PEGylated AgNPs +EAC	3.44 ± 0.008	4.65 ± 0.013
One way ANOVA with EAC (P< 0.05)	0.017	-
One way ANOVA without EAC (P< 0.05)	0.016	0.062
Two way ANOVA without EAC (P< 0.05)	0.019	

The values are mean ± SD of six animals

Figure 41

Effect of ethanolic leaves extract and PEGylated AgNPs on the levels of Vitamin E in the liver of control and EAC induced Swiss albino mice



The values are mean ± SD of six animals
Means which are followed by different superscripts differ significantly at P < 0.05
Means which are followed by same superscripts do not differ significantly at P<0.05

Vitamin E is a potent biological antioxidant which acts as a membrane bound antioxidant to protect the cell against lipid peroxidation and plays a vital role in inhibiting carcinogenesis. It plays an important role in free radical scavenging (Parks and Traber, 2000). In EAC induced mice a decreased level of Vitamin E was noted which was due to excessive exploitation of this antioxidant for quenching enormous free radicals generated due to tumor burden.

In mucosal tissue of colorectal cancer a decreased level of Vitamin E was noted (Skrzydlewska *et al.*, 2005). Administration of green tea showed a significant increase in Vitamin E in the liver and kidney of rats induced with ammonium metavanedate toxicity (Soussi *et al.*, 2006). Sarada Devi *et al.* (2011) reported that an increase in the levels of Vitamin E and A was observed in the liver tissue of Balb/c mice on treatment with ethyl acetate fraction of *Cynodon dactylon.*

4.5.2.3 Effect on reduced glutathione (GSH)

The level of reduced glutathione was significantly elevated by the intraperitoneal administration of ethanolic leaves extract and PEGylated AgNPs when compared to that of the vehicle control groups. The level of GSH was increased in silymarin treated mice in 15 days and 60 days of treatment period when compared to the vehicle control paraffin oil. PEGylated AgNPs treated mice showed more significant GSH level than that of the ethanolic leaves extract and standard antioxidant silymarin treated group in both the treatment periods.

The level of GSH in liver of EAC cells bearing mice was significantly reduced in comparison with normal control and all the experimental groups. Coadministration of ethanolic leaves extract and PEGylated AgNPs to EAC induced mice showed significant increase in GSH levels as shown in Table 13 and Figure 42.

Table 13

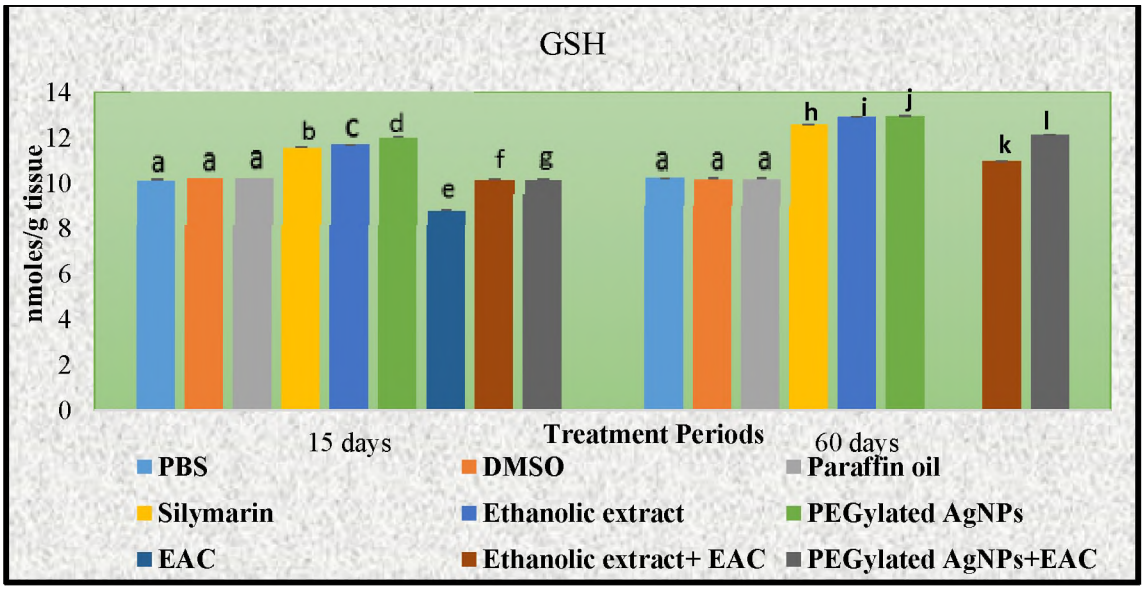
Levels of Reduced glutathione in hepatic tissues of control and experimental Swiss albino mice

Groups	Reduced glutathione (nmoles/ g tissue)	
	15 Days	60Days
PBS	10.12 ± 0.015	10.20 ± 0.007
DMSO	10.17 ± 0.019	10.18 ± 0.042
Paraffin oil	10.17 ± 0.017	10.18 ± 0.048
Silymarin	11.53 ± 0.008	12.58 ± 0.011
Ethanolic extract	11.64 ± 0.026	12.93 ± 0.015
PEGylated AgNPs	11.96 ± 0.013	12.96 ± 0.012
EAC	8.76 ± 0.026	-
Ethanolic extract +EAC	10.14 ± 0.008	10.98 ± 0.431
PEGylated AgNPs +EAC	10.16 ± 0.016	12.13 ± 0.021
One way ANOVA with EAC (P< 0.05)	0.019	-
One way ANOVA without EAC (P< 0.05)	0.018	0.162
Two way ANOVA without EAC (P< 0.05)	0.018	

The values are mean ± SD of six animals

Figure 42

Effect of ethanolic leaves extract and PEGylated AgNPs on the levels of GSH in the liver of control and EAC induced Swiss albino mice



The values are mean ± SD of six animals

Means which are followed by different superscripts differ significantly at P < 0.05

Means which are followed by same superscripts do not differ significantly at P<0.05

Glutathione is known as body's master antioxidant. Highest concentration of glutathione was present in the liver which plays a major role in detoxification process. It plays an important role in body's defense system by neutralizing and eliminating free radicals. It facilitates the alleviation of oxidative stress (http://www.essentialgsh.com). Coadministration of ethanolic leaves extract and PEGylated AgNPs caused a significant increase in the level of GSH in EAC induced mice. Of which PEGylated AgNPs showed a better effect than ethanolic leaves extract.

Girija and Asha (2011) reported that a significant decrease in the levels of SOD, CAT, GPX and GSH in benzo (a) pyrene induced lung cancer in Swiss albino mice. The saponin fraction of *Euphorbia nerifolia* caused an increase in the levels of hepatic glutathione in CCl_4 induced hepatotoxicity in rats (Bigoniya and Rana, 2010). An increase in the levels of non enzymic antioxidants such as glutathione, Vitamin E and C were found in rats treated with the methanolic leaf extract of *Cassia tora.*

A depletion in the level of GSH leads to peroxidative injury due to cellular defense against reactive oxygen species. In addition to free radical scavenger GSH plays as a substrate for GPX and GST. A significant decrease in the level of GSH was observed in EAC cells induced mice which was due to excess utilization of GSH to neutralize the free radicals. Coadministration of ethanolic leaves extract and PEGylated AgNPs to EAC induced mice caused an increase in the level of GSH.

The non enzymic antioxidants such as Vitamin A, E and reduced glutathione are known as effective antioxidants in living organisms. These non enzymic antioxidants protects the cell and tissue by scavenging the generated ROS in the body. In our present research work a significant diminution in the level of non enzymic antioxidants was observed in EAC induced mice which was due to the excessive utilization of these antioxidants to combat the oxidative stress due to tumor burden. A significant increase in the level of these antioxidants was observed in mice treated with PEGylated AgNPs and ethanolic leaves extract individually and in EAC propagated mice. The PEGylated AgNPs administered group showed a better effect than the ethanolic leaves extract treated group.

The PEGylated AgNPs showed a better enhancement in the activities of endogenous (enzymic) and exogenous (non enzymic) antioxidants which effectively scavenges the free radicals by preventing and repairing the damages caused due to the excess production of ROS and RNS thereby enhances the immune defense and lowers the risk of tumor burden.

4.5.6 Effect of PEGylated AgNPs on the Rate of Lipid Peroxidation

Lipid peroxidation is a process were cellular damage occurs due to the bargaining of electrons from the lipids present in the cell membrane by free radicals. During LPO, oxidative dregradation of lipid occurs. Increased level of LPO would cause degeneration of tissues. The end product of LPO *i.e* malondialdehyde (MDA) cause damage to the membrane. It was higher in carcinomatous tissue than in non-diseased organs. MDA plays an important role as tumor promoter and acts as an inhibitory agent for protective enzymes (Ristow and Zarse 2010).

Table 14

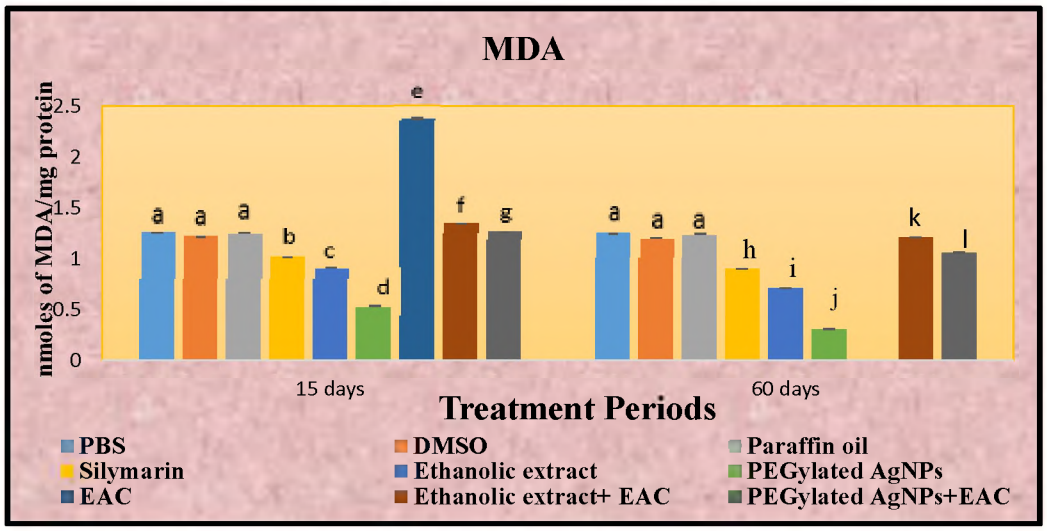
Levels of hepatic MDA in control and experimental Swiss albino mice

Groups	nmoles of MDA/mg protein	
	15 Days	60Days
PBS	1.25 ± 0.024	1.24 ± 0.016
DMSO	1.21 ± 0.008	1.19 ± 0.013
Paraffin oil	1.24 ± 0.015	1.23 ± 0.024
Silymarin	1.01 ± 0.008	0.9 ± 0.012
Ethanolic extract	0.90 ± 0.012	0.71 ± 0.014
PEGylated AgNPs	0.52 ± 0.040	0.31 ± 0.029
EAC	2.37 ± 0.014	-
Ethanolic extract+ EAC	1.34 ± 0.011	1.21 ± 0.057
PEGylated AgNPs +EAC	1.26 ± 0.027	1.06 ± 0.016
One way ANOVA with EAC (P< 0.05)	0.023	-
One way ANOVA without EAC (P< 0.05)	0.022	0.195
Two way ANOVA without EAC (P< 0.05)	0.022	

The values are mean ± SD of six animals

Figure 43

Effect of ethanolic leaves extract and PEGylated AgNPs on the levels of MDA in the liver of control and EAC induced Swiss albino mice



The values are mean ± SD of six animals
Means which are followed by different superscripts differ significantly at P < 0.05
Means which are followed by same superscripts do not differ significantly at P<0.05

Table 14 and Figure 43 show the levels of lipid peroxide (MDA) in the hepatic tissue of control and experimental groups of mice. Administration of ethanolic leaves extract, PEGylated AgNPs and silymarin significantly reduced the level of MDA when compared to the control groups in both the treatment periods. The level of MDA was significantly decreased in PEGylated AgNPs treated mice followed by ethanolic leaves extract and silymarin treated groups. A significant increase in the levels of MDA was observed in EAC cells bearing mice when compared to all the other control and experimental animals. Coadministration of ethanolic leaves extract and PEGylated AgNPs to EAC cells induced mice showed significant decrease in the level of MDA when compared to EAC control mice in 15 days treatment period. Coadministration of ethanolic leaves extract and PEGylated AgNPs to EAC cells induced tumor mice showed significant decrease in the levels of MDA in 60 days treatment period when compared to 15 days treatment period.

Our data clearly showed a significant decrease in the level of MDA in mice supplemented with ethanolic leaves extract and PEGylated AgNPs individually and in

EAC cells induced mice. The rate of inhibition of lipid peroxidation was found to be to fold which enhanced the antioxidative defense mechanisms and prevented the formation of excessive free radicals and confirmed the antilipid peroxidative role of ethanolic leaves extract and PEGylated AgNPs in EAC induced mice. Antilipid peroxidative role was found to be more significant in PEGylated AgNPs supplementation than that found in ethanolic leaves extract treated mice.

Lung-Yuan *et al.* (2011) reported that the ethanolic extract of *Dendrobium tosaense* and *Ephemerantha fimbriata* significantly decreased the level of MDA in carbon-tetrachloride induced acute liver injury in mice. The level of MDA was decreased in CCl_4 induced rats on administration of the whole leaf juice of *Colocasia esculenta* (Patil and Ageely, 2011).

Administration of ethanolic leaves extract and PEGylated AgNPs significantly reduced the level of MDA, due to the reduced lipid peroxidation and elevation of tissue antioxidant defense enzyme activity. This signifies that both the ethanolic leaves extract and PEGylated AgNPs could decrease the formation of free radicals and increase the free radical scavenging mechanism. EAC cells induced mice showed an increase in the level of MDA which is due to the enhancement in lipid peroxidation and excess production of free radicals caused due to defect in antioxidant defense mechanism. From the observations it is clearly manifested that the mechanism of hepato protection was due to their effective antioxidant potential.

4.5.7 Effect of PEGylated AgNPs on the Histological Status of Hepatocytes of Control and EAC Challenged Swiss albino Mice

The histological status of liver was observed under light microscope to detect the effect of ethanolic leaves extract and PEGylated AgNPs in EAC cells induced mice. The structural integrity of liver cells were shown in Plate 8. The control groups (PBS, DMSO, Paraffin oil) showed normal cellular architecture with intact central vein and sinusoids, normal portal triad and kupffer cells. The hepatic cells of mice treated with silymarin showed normal cellular architecture with intact central vein and sinusoids, normal portal triad and kupffer cells. There was no evidence of cell necrosis. Histopathological

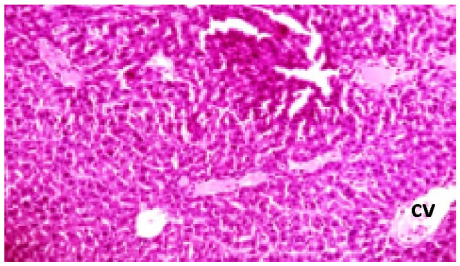
observations in the liver of ethanolic leaves extract and PEGylated AgNPs treated mice were comparable with the control and silymarin without any structural changes in the liver morphology.

The EAC induced mice showed severe necrosis, surrounding fibrosis, perivenular inflammation and vacuole formation. However, mice treated with silymarin, ethanolic leaves extract and PEGylated AgNPs showed reduced vacuole formation and inflammation and almost normal hepatocellular architecture. Histopathological examination showed a protective effect of ethanolic leaves extract and PEGylated AgNPs against the hepatotoxicity induced by EAC cells. The prevention of necrosis by the treatment of ethanolic leaves extract and PEGylated AgNPs may be due to antilipid peroxidation, diminution of oxidative stress and free radicals formation.

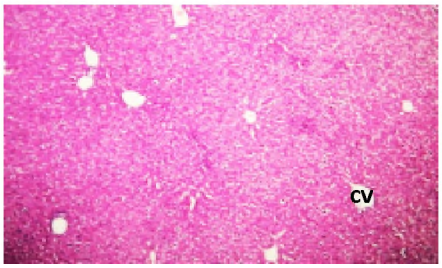
Plate 8

Histological status of the liver of control and experimental Swiss albino mice (40X)

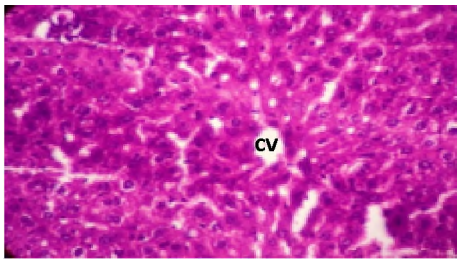
EAC



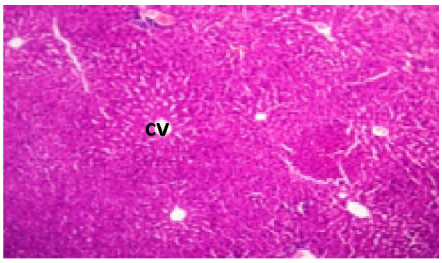
PBS



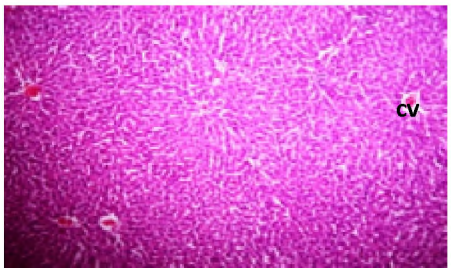
DMSO



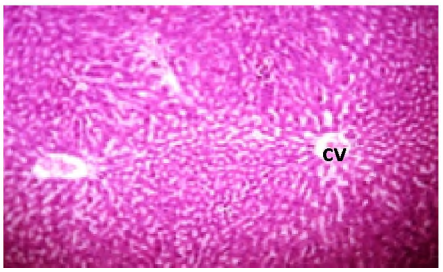
Paraffin oil



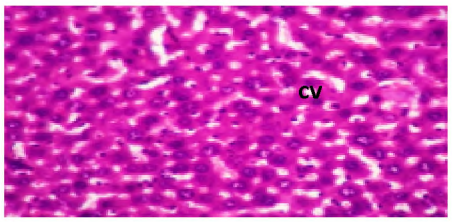
Silymarin



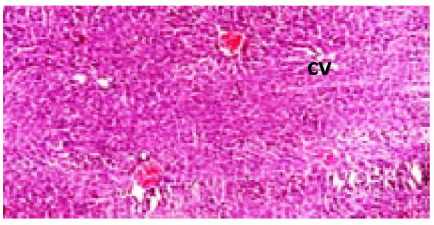
Ethanolic extract



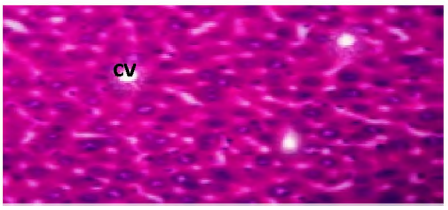
PEGylated AgNPs



Ethanolic extract + EAC



I) PEGylated AgNPs + EAC



cv – central vein

D'Archivio *et al.* (2008) also reported these obvious changes in the hepatic architecture of EAC induced mice due to the toxic effect exerted by the generation of free radicals, which resulted in the excess production of malondialdehyde and conjucated dienes leads to deleterious effect on the membranous components of hepatocytes

Nanoparticles remarkably suppressed pulmonary metastasis. Smears treated with silver nanoparticles showed very few pleomorphic cells with hyperchromatic nuclei and significant reduction in malignant cell clumps against DLA cells (Sriram *et al.*, 2010). The extract of *Zizypus* leaves diminished pathological alterations in the liver hepatocytes induced by EAC cells (Hassan and Abdel-Gawad, 2010). Sakthivel *et al.* (2012) reported reduced vacuole formation and inflammation and normal hepatocellular architecture in mice treated with *Acacia nilotica* extract against DAL cells induced mice.

In the present study, the histological examination of the liver of EAC induced Swiss albino mice showed marked changes indicating the toxic effects in tumor burden. These effects were reverted back as in control mice on treatment with PEGylated AgNPs and ethanolic leaves extract.

In conclusion, PEGylated AgNPs were effective in inhibiting the tumor growth in EAC models when compared to the ethanolic leaves extract. Life span, both the *in vitro* and *in vivo* biochemical parameters and histological studies supported their antioxidative and antitumorigenic properties.

Our study, thus optimized the method for the green synthesis of silver nanoparticles and its functionalization using PEG. Characterization of the functionalized silver nanoparticles revealed their ideal size and surface charge and other characters required for druggability, including the drug release profile at different pH conditions. The PEGylated silver nanoparticles showed good bioactivity by enhancing the antioxidative role and by inhibiting lipid peroxidation. Further study and human trial will be of use in validating the therapeutic efficacy of PEGylated AgNPs as a potential antitumorigenic agent. The results of the study were summarized and the conclusions drawn therein are presented in the next chapter.