

Antiproliferative effect of Phytosome complex of Methanolic extact of *Terminalia Arjuna* bark on Human Breast Cancer Cell Lines (MCF-7)

Sharma Shalini^{1*}

Roy Ram Kumar²

Shrivastava Birendra³

¹Sunder Deep Pharmacy College,
NH-24, Sunder Deep Nagar,
Dasna, Ghaziabad, U.P., India.

²D.V. Institute of Pharmacy &
Medicine, Karnal, Haryana, India.

³School of Pharmaceutical
Sciences, Jaipur National
University, Jaipur, Rajasthan, India.

Corresponding Authors:

Mobile No: +917503747010.

Email:

shalinisharma23@yahoo.com

Fax No: +91122765254

Abstract:

Methanolic extract of *Terminalia arjuna* Roxb of family combretaceae is rich in flavonoids content which are responsible for its antiproliferative activity but these bioactive constituents have poor oral and topical absorption either due to the large molecular weight or poor miscibility with lipids. These poorly soluble herbal extracts can be converted into lipid compatible molecular complexes called phytosomes by binding individual constituents of the herbal extract to phosphatidylcholine. Phytosomes are known to show improved oral and topical absorption followed by enhanced activity as compared to pure herbal extracts. This study was aimed at preparing methanolic extract of *Terminalia arjuna* bark and *Terminalia arjuna* bark Extract Phytosome and investigating their antiproliferative activity on human MCF-7 cell line by MTT assay. Comparison of the antiproliferative activity was done with with quercetin and its phytosomes. IC₅₀ of methanolic extract of *Terminalia arjuna* bark and *Terminalia arjuna* bark Extract Phytosome against cancer cell lines MCF-7 was found to be 25µg/ml and 15µg/ml respectively whereas that of quercetin and its phytosomes was found to be 2µg/ml and 0.7µg/ml respectively. The results suggests that *Terminalia arjuna* bark Extract Phytosome & quercetin phytosomes are active pharmacologically and exerts more antiproliferative effect on MCF-7 cells as compared to pure methanolic extract of plant and pure quercetin respectively.

Keywords: *Terminalia arjuna* bark, antiproliferative activity, phytosome, MCF 7, MTT assay, adenocarcinoma

1. INTRODUCTION:

Terminalia arjuna Roxb of family Combretaceae is an evergreen large deciduous tree widely found throughout India [1]. The *T. arjuna* (TA) has been known for its broad spectrum activities such as hypocholesteremic, hypolipidemic, anticoagulant, antihypertensive, antithrombotic, dropsy, diuretics, anti-infective and anti-asthmatic. TA is used in the treatment of cardiac failure, rheumatoid arthritis and cancer [2-4].

Methanolic extract of the TA bark is rich in flavonoids which are known to be the most effective anticancer agent. Flavonoids are known to be degraded in gastrointestinal tract after oral

absorption which results in the poor bioavailability [3, 5]. Moreover flavonoids are high molecular weight molecules too large to be absorbed by simple diffusion [6]. Thus Phytoconstituents despite having excellent bioactivity *in vitro*, demonstrate less or no *in vivo* actions due to their poor lipid solubility or improper molecular size or both resulting in poor absorption and poor bioavailability. Lipid solubility and molecular size are the major limiting factors for molecules to pass the biological membrane and to be absorbed systematically following oral or topical administration. Some phytoconstituents are destroyed in the gastric environment when taken orally [7].

With a view to enhancing bioavailability of herbal drugs phytosome have been prepared which is an innovative delivery system composed by a natural active ingredient and a phospholipid and in which hydrophilic constituents of the herbs are surrounded and bound by phospholipids [8]. These lipophilic complexes developed by Indena increases the absorption and utilization of standardized herbal extracts by incorporation of phospholipids.

Phytosomes are advanced herbal products produced by binding individual component of herbal extracts to phosphatidylcholine resulting in a dosage form that is better absorbed and thus produces better results than the conventional herbal extracts [9]. Phosphatidylcholine is known to regulate the fluidity and permeability of the cell membrane. Phosphatidylcholine because of its lipophilic nature are known to cross cell membrane and thus increases the oral and topical absorption of herbal extract which are known to have poor bioavailability [10, 11].

The present study was designed to assess the preparation of *Terminalia arjuna* bark Extract (TBE) & its phytosomes (TBEP) and evaluate its antiproliferative activity on human breast cancer cell line MCF-7 by using MTT assay. Quercetin (QC) and its phytosomes (QCP) were taken as reference standards. The MTT assay is useful in determination of cellular proliferation, viability and activation. MTT assay is one of the most widely used methods for preliminary screening of different substances in cell cultures [12]. This technique measures the ability of living cells to reduce the tetrazolium salt MTT to formazan by NADH reductase and other enzymes which can be measured spectrophotometrically. It is well established that there is a linear relationship between the number of live cells and the amount

of formazan produced thus validating this methodology to measure cellular metabolic activity. MTT enter the cell by endocytosis and is converted to formazan. The amount of formazan reflects the reductive potential of the cytoplasm and the cell viability [13].

2. MATERIALS AND METHODS

All the reagents used were of analytical grade. Hydrogenated Phosphatidylcholine (Phospholipon 90H) was a gift sample from Lipoid, Germany. Phospholipid contains fatty acids (stearic acid and palmitic acid, 98%) and sum of the unsaturated fatty acids (oleic acid, linoleic acid, 2%). TA bark was purchased from a local supplier and authenticated by Dr. H. B. Singh, Chief Scientist and Head, Raw Material Herbarium and Museum (RHMD), National Institute of Science Communication and Information Resources (NISCAIR).

Preparation of TBE

Coarsely powdered bark of TA was placed in a borosilicate glass container with methanol and allowed to stand at room temperature for a period of at least three days with frequent agitation until all the soluble matter has been dissolved. The mixture was then strained and the marc pressed. The combined liquid was clarified by filtration. The solvent was evaporated and the extract so obtained was used to prepare phytosomes.

Preparation of TBEP

10g of TBE was dissolved in 200 ml of a mixture of 6 parts of methylene chloride and 1 part of methanol. 15g of phospholipid containing hydrogenated phosphatidylcholine was added in small portions. When dissolution was complete, the solvent was distilled off under vacuum to small

volume. The residue so obtained was diluted with 200 ml of methylene chloride and filtered. Solvent was evaporated to small volume and the mixture was diluted with 300 ml of n-hexane. On dilution with n-hexane, the product was precipitated in the form of light brown solid which was air dried. The product so obtained was completely soluble in non-polar solvents [14].

Preparation of QCP:

The complex was prepared with quercetin and phospholipid at a molar ratio of 1:2 according to a patented procedure [15]. 3.08 g of QC was suspended in 100 ml methylene chloride. 8 g of hydrogenated phosphatidylcholine was added to the suspension and the mixture was heated to complete dissolution. The solution was concentrated to a volume of 20 ml and then diluted with 200 ml of n-hexane under stirring. The precipitated product was filtered and air dried. The resultant yellow colored complex was kept in an amber colored glass bottle, and stored.

TEM of TBEP & QCP

In order to prepare sample, one drop of TBEP was added in Para film. Copper grid was dipped into this sample for one minute. For negative staining a drop of 2% phosphotungstic acid was added in para film and grid was immediately dipped into this solution and allowed to float for 30s to 1 min. Excessive solution was removed by using filter paper. This stained film was then viewed on transmission electron microscope (Morgagni 268-D Fei, Holland) at All India Institute of Medical Sciences New Delhi. Samples was observed at various magnifications ranging from 5000-75000 magnifications by applying 90 KV energy.

NMR Spectroscopic analysis of the TBE, TBEP, QC and QCP

The ¹H NMR spectra of TBE, TBEP, QC and QCP were recorded using BRUKER AVANCE II 400 NMR

Spectrometer at Sophisticated Analytical Instrumentation Facility, Punjab University Chandigarh. An amount of 50 mg of each sample was dissolved in 0.8 ml of dimethylsulphoxide (DMSO) and each sample was measured and recorded.

Cell proliferation assay (MTT) of TBE, TBEP, QC and QCP

Effect of TBE as well as TBEP on cell proliferation of MCF-7 human cell line of breast adenocarcinoma was carried out using MTT assay. The human tumor cytotoxicity was determined following protocols established by National Cancer Institute [16]. MCF-7 cell lines was cultured in RPMI 1640 medium (Bio Whittaker) containing 20% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin. For the determination of cell viability the cells were plated at the density of 5×10³ cells/well and treated with 0.01, 0.1, 1.0, 10 and 100µg/ml concentration of TBE and TBEP for 96 hrs and incubated with 1% DMSO as solvent control. The absorbance of the solutions at different concentration was recorded at 550 nm and the cell numbers were analyzed. IC₅₀ (Concentration of compound required to inhibit 50% cell growth) was determined by plotting a graph of concentration of compound vs. percentage cell inhibition. A line drawn from 50% value on the Y axis meets the curve and interpolate to the X axis. The X axis value gives the concentration of compound. Percentage inhibition of novel compounds against all cell lines was calculated using the following formula

$$\% \text{ cell survival} = [(A_t - A_b) / (A_c - A_b)] \times 100$$

Where, A_t = Absorbance of Test,

A_b = Absorbance of Blank (Media),

A_c = Absorbance of Control (cells)

$$\% \text{ cell inhibition} = 100 - \% \text{ cell survival}$$

MTT assay was also carried out for reference standards QC and QCP in the similar manner.

3. RESULTS

TA Sample was identified as *Terminalia arjuna* (Roxb. Ex DC.) Weight & Am. wide ref. no. NISCAIR/RHMD/Consult/-2011-12/1789/89. The obtained yield of TBE was found to be 0.3%. TBE was obtained as dark brown, shining crystals. A TEM study of TBEP shows spherical molecules with size range of 31.02 nm to 80.17 nm. As observed TBEP complexes were well identified perfect spheres and seen in disperse and aggregate collection as shown in Fig. 1. QCP were also perfect sphere but comparatively smaller in size as shown in Fig 2. The effect of TBE and TBEP on the growth of MCF-7 cell lines was examined by MTT assay. IC₅₀ was found to be 15 µg/ml and 25 µg/ml for TBEP and TBE respectively in MCF-7 cell line. QC and QCP were taken as reference standards and their IC₅₀ was found to be 7 µg /ml and 0.2 µg/ml. Results are summarized in Table 1.

Sample	IC 50 (micrograms/ml)
<i>Terminalia arjuna</i> Bark Extract (TBE)	25
<i>Terminalia arjuna</i> Bark Extract Phytosomes (TBEP)	15
Quercetin (QC)	2
Quercetin Phytosomes (QCP)	0.7

Table 1: Comparative Antiproliferative Activity of TBE, TBEP, QC and its Phytosomal Complex

¹H NMR analysis of TBE and TBEP Spectra obtained from ¹H NMR analysis of TBE, TBEP and Hydrogenated phosphatidylcholine (HP) are as follows:

For TBE: ¹H NMR (DMSO) δ 7.70 (1H, d), 3.69-3.09 (17H, m), 2.50 (1H, q), 2.08 (4H, s), 1.17 (1H, d), 0.85 (1H, m) (Fig.3a).

For TBEP: ¹H NMR (DMSO) δ 7.63 (3H, s), 4.31 (1H, s), 4.01 (2H, d), 3.71-3.30 (4H, m), 2.50 (1H, quintet), 2.19 (1H, t), 2.08 (1H, s), 1.50 (2H, s), 1.17 (20H, s), 0.78 (3H, m) (Fig. 3b).

For Hydrogenated phosphatidylcholine : ¹H NMR (DMSO) δ 4.85 (1H, s), 4.08-3.96 (1H, m), 3.83-3.62 (2H, m), 3.38 (1H, s), 3.07 (15H, s), 2.95 (8H, d), 2.27 (1H, s), 1.91 (2H, m), 1.26 (2H, s), 0.95 (23H, s), 0.57 (3H, s) (Fig. 3c)

For QC: FTIR (KBr) cm⁻¹ 3404, 1356 (O-H), 2973, 911, 843 (Ar-H), 1689 (C=O), 1605 (C=C of Ar), 1002, 1198 (C-O). ¹H NMR (DMSO) δ 12.19 (1H, s), 10.22 (1H, s), 8.44 (2H, s), 7.70-7.68 (1H, m), 7.51 (1H, dd), 6.82 (1H, d), 6.30 (1H, d), 6.13 (1H, d). (Fig, 3d)

For QCP: FTIR (KBr) cm⁻¹ 2904, 1483, 1375 (CH₂ and CH₃), 2820, 1350 (O-H), 1749 (C=O), 1599 (C=C of Ar), 1269 (P=O), 1226 (P-O), 1190, 1007 (C-O), 1124 (C-N), 917, 845 (Ar-H). ¹H NMR (DMSO) δ 12.30 (1H, s), 10.47 (1H, s), 9.28 (1H, s), 9.04 (1H, s), 8.85 (1H, s), 7.67 (1H, d), 7.50 (1H, dd), 6.82 (1H, d), 6.32 (1H, d), 6.12 (1H, d), 3.89 (1H, t), 3.60 (1H, t), 3.36 (6H, s), 3.16 (1H, s), 2.50 (1H, t), 2.15 (1H, m), 1.49 (1H, s), 1.19 (8H, s), 0.80 (1H, t). (Fig 3e)

4. DISCUSSION

Transmission Electron micrograph of TBEP appeared as amorphous particles as the drug is uniformly dispersed in phospholipids and formed the structure of spherical particles. The formation of molecular complex is confirmed by ¹H NMR spectroscopy study by comparing the spectra of the constituents with those of the phytosome complex. DMSO having high dielectric constant used to dissolve the phytosome complex is known to cleave the complex. Complexes dissolved in these types of solvent with high dielectric constant shows spectra which are a summary of spectra registered for individual compound. In the ¹H-NMR

spectrum of the complex the signals from the protons of the lipid chain are well evident as well as the broadening of the band of methyl group of choline showing that this moiety is involved in the complex formation. The polar head of the phospholipids and active sites of the complex are involved in complex formation while the fatty acid moieties are free so the resulting complex is lipophilic in nature. The lipophilic nature is further confirmed by solubility of the complex in nonpolar and aprotic solvents as compared to the pure extract. Standard extract is initially insoluble in chloroform, ethyl acetate and benzene but it becomes extremely soluble in these solvents after forming a complex with phospholipid. This change in physical and chemical properties is due to formation of true stable complex as is evident from the $^1\text{H-NMR}$. Hydrogenated phosphatidylcholine contained in phospholipid being a selective carrier allows the molecule to have both an improved crossing through the horny layer of the skin and better interaction with cell and bacterial walls. Due to surfactant properties of phospholipid, the product is better adhered to the mucosal surface and makes it easy for the complex product to better interact with cell structure. TBEP & QCP thus shows better pharmacological activity as compared to TBE & QC respectively and the same is evident by the IC_{50} values as shown in table 1.

Presence of polyphenols, flavonoids, tannins and saponins are responsible for the antiproliferative activity against breast adenocarcinoma. Hydrolysable tannin named casuarinin isolated from the bark of TA is reported to inhibit breast cancer cell growth by inducing apoptosis and cell cycle arrest in human breast adenocarcinoma MCF 7 cells [17-19]. Methanolic extract of the TA bark is reported to exhibit antimutagenic activity

[2-3]. It also inhibits the growth of human fibroblasts, osteocarcinoma, glioblastoma and is reported to be effective in reducing the DNA damage caused by 4-nitroquinoline [20, 21]. In vitro antiproliferative activity of the TBE as well as TBEP was determined against breast adenocarcinoma MCF-7 cells line. In our experiment TBE exhibited potent antiproliferative activity against the MCF-7 cells (Graph. 2). The next step in our investigation was to test antiproliferative activity of TBEP and compare its antiproliferative activity with TBE. TBE is rich in flavonoid content. Flavonoids detected from its bark are arjunolone, flavones, baicalein, quercetin, kempferol and pelargonidin. Flavonoids possess effective antiproliferative activity against cancer cells [22-25]. Might be these flavonoids are responsible for antiproliferative activity. Another study also suggests that elagitannin named arjunin obtained from bark extract of TA is responsible for antiproliferative activity [26]. The effect of TBE and TBEP on the growth of MCF-7 cell lines was examined by MTT assay. Dose response curve constructed between the range 0.01–100 $\mu\text{g/ml}$ for TBE and TBEP expresses decreasing number of viable cells with increasing concentration of compounds aliquots. Calculation of IC_{50} value was done using Graph Pad Prism Software, version 5. The susceptibility of cells to the TBE and TBEP was characterized by IC_{50} (Graph 1 & 2). Result indicates that the antiproliferative effect steadily strengthens with increase in the concentration of TBE and TBEP. TBE and TBEP exhibit activity in breast cancer cell line. IC_{50} was found to be 15 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$ for TBEP and TBE respectively in MCF 7 cell line. Crude extract with IC_{50} value less than 30 $\mu\text{g/ml}$ is considered to be active as per American Cancer Institute [27]. TBEP were found to be more active against breast cancer

cell lines MCF-7 with lower IC_{50} as compared to TBE.

A numbers of studies have demonstrated that QC inhibits lipid peroxidation effectively by free radical scavenging and/or chelation of transition metal ions and is already approved for use in Human in leukemia blasts [28]. QC and its phytosomal complex have definite potential to become a chemotherapeutic agent for human cancer. Inhibition of the activities of enzymes like EGF receptor tyrosine kinase and protein kinase and glutathione reductase might be an important factor in the occurrence of apoptosis of tumor cells by QC [24, 29-30]. The results suggests that QC exerts antiproliferative effect on MCF-7 cells but the activity of QCP was more with lower IC_{50} as compared to that of QC (Graph 3&4). This further strengthens the fact that phytosomes are the herbal products wherein the individual components of herbal extracts binds to phosphatidylcholine and produces a dosage form which shows better pharmacological activity and bioavailability as compared to its pure molecular adducts [6].

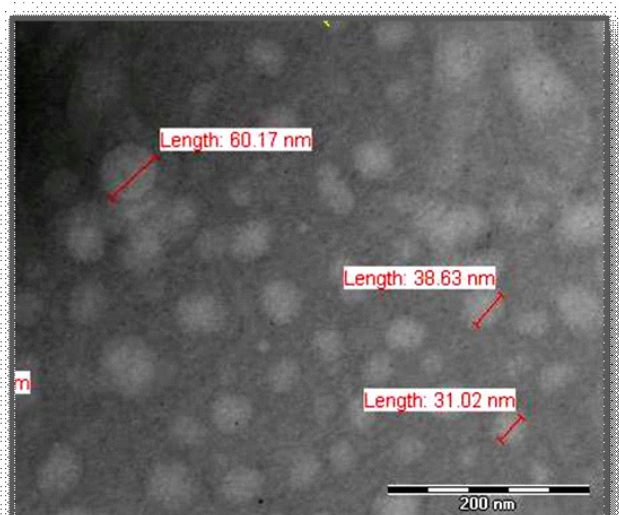


Figure 1: TEM of TBEP

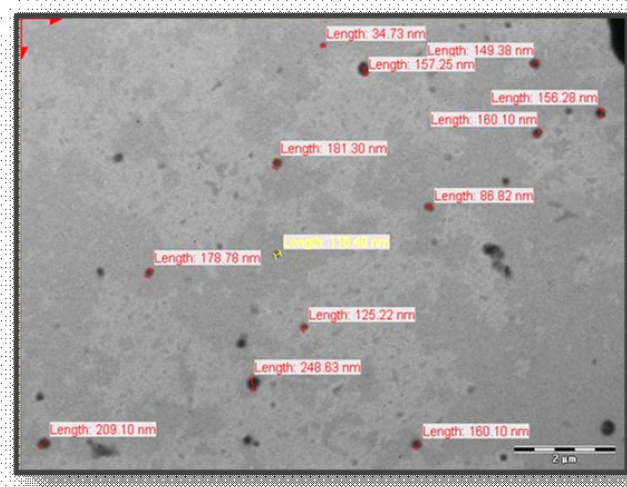
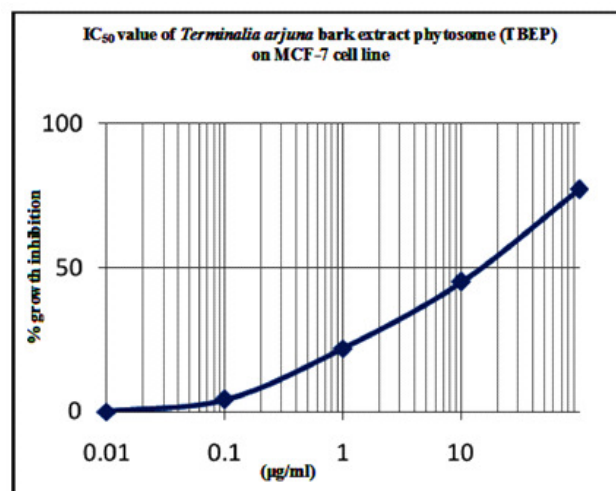
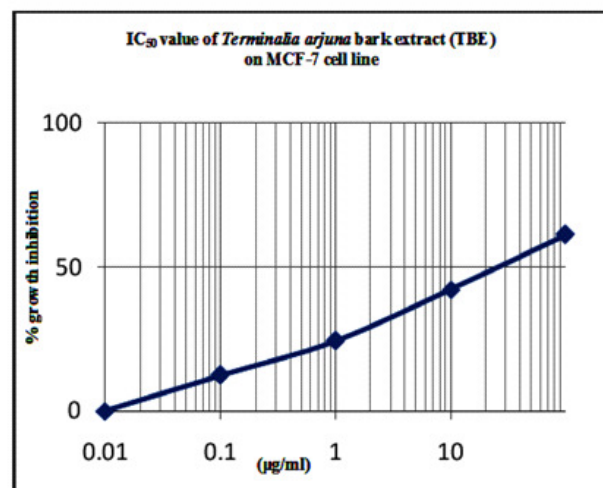


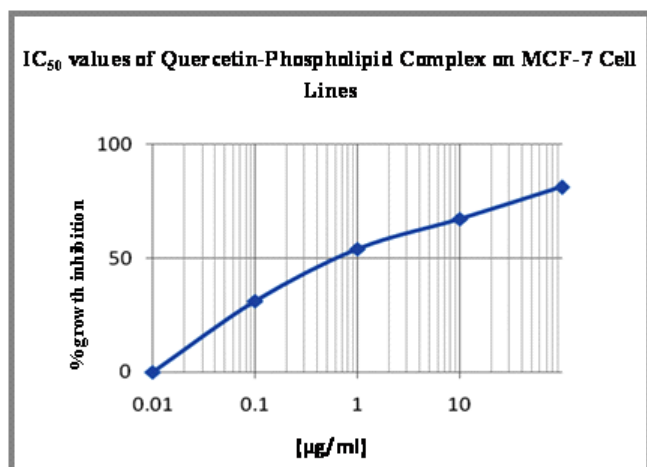
Figure 2: TEM of QCP



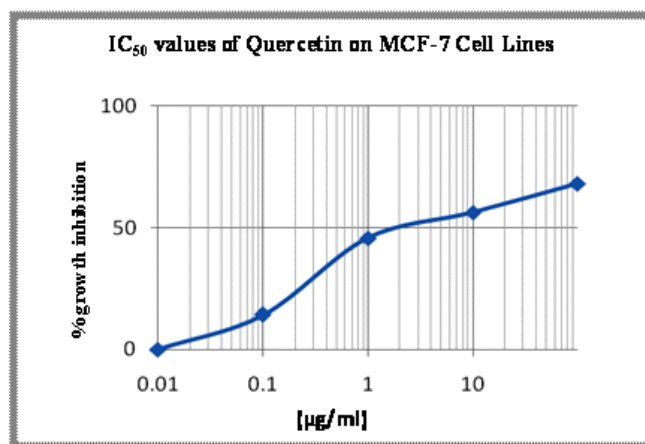
Graph 1-Activity of *Terminalia arjuna* bark extract phytosome (TBEP) against MCF-7 cell lines



Graph 2-Activity of *Terminalia arjuna* bark extract (TBE) against MCF-7 cell



Graph 3: Activity of Quercetin-Phytosome against (QCP) against MCF-7 cell lines by MTT assay



Graph 4: Activity of Quercetin (QC) against MCF-7 cell lines by MTT assay

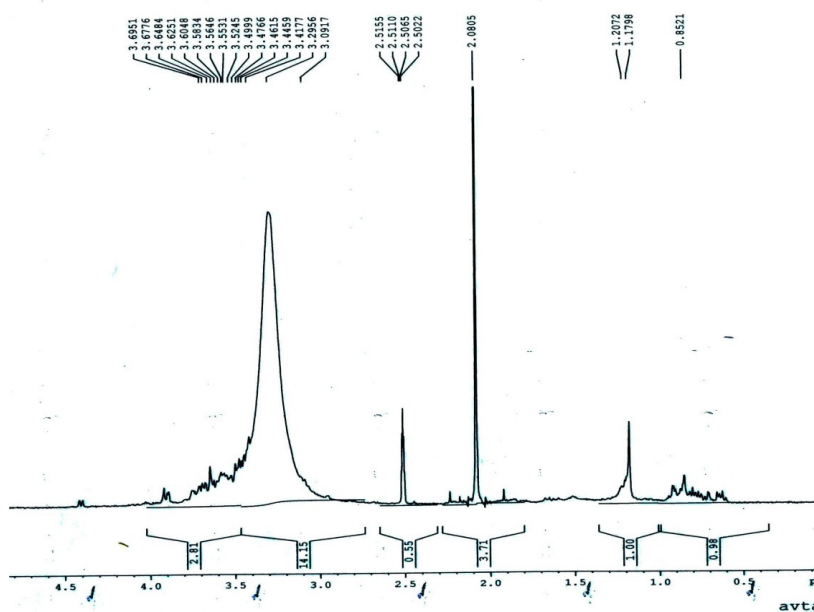


Figure 3a: NMR Spectrogram of *Terminalia arjuna* bark extract (TBE)

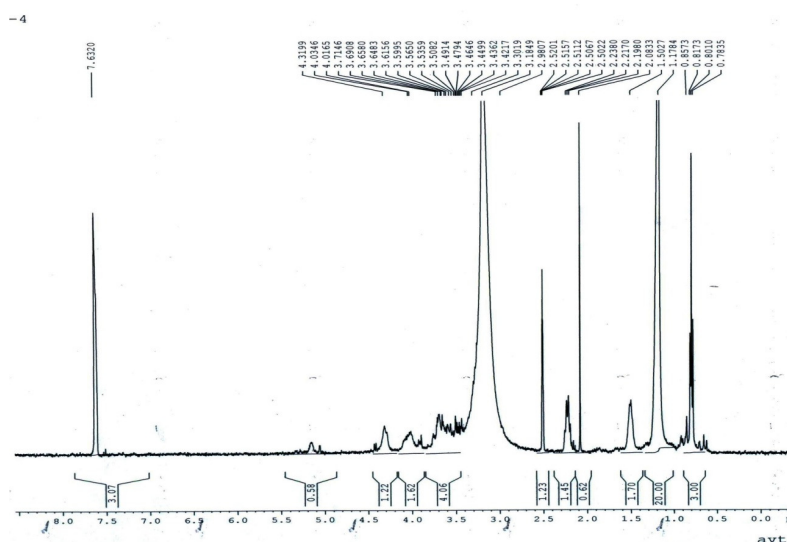


Figure 3b: NMR Spectrogram of *Terminalia arjuna* bark extract phytosome (TBEP)

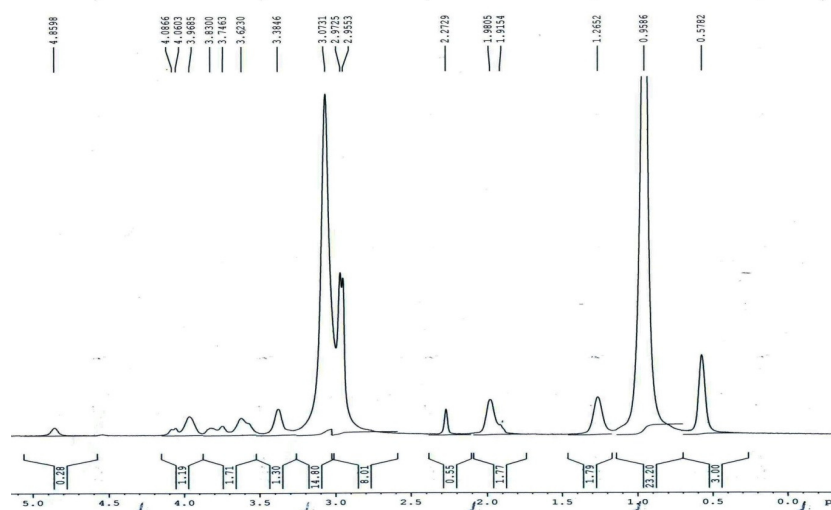


Figure 3c: NMR Spectrogram of Phospholipon 90H (Hydrogenated Phosphatidylcholine)

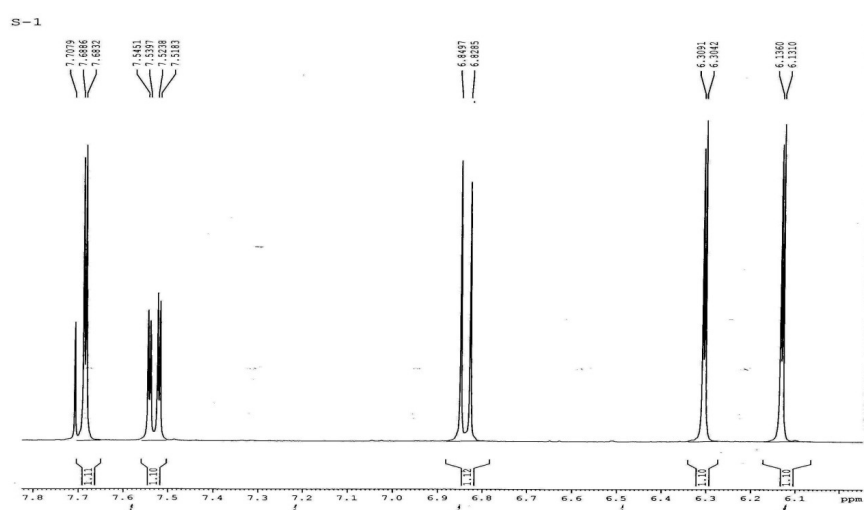


Figure 3d: NMR Spectrogram of Quercetin (QC)

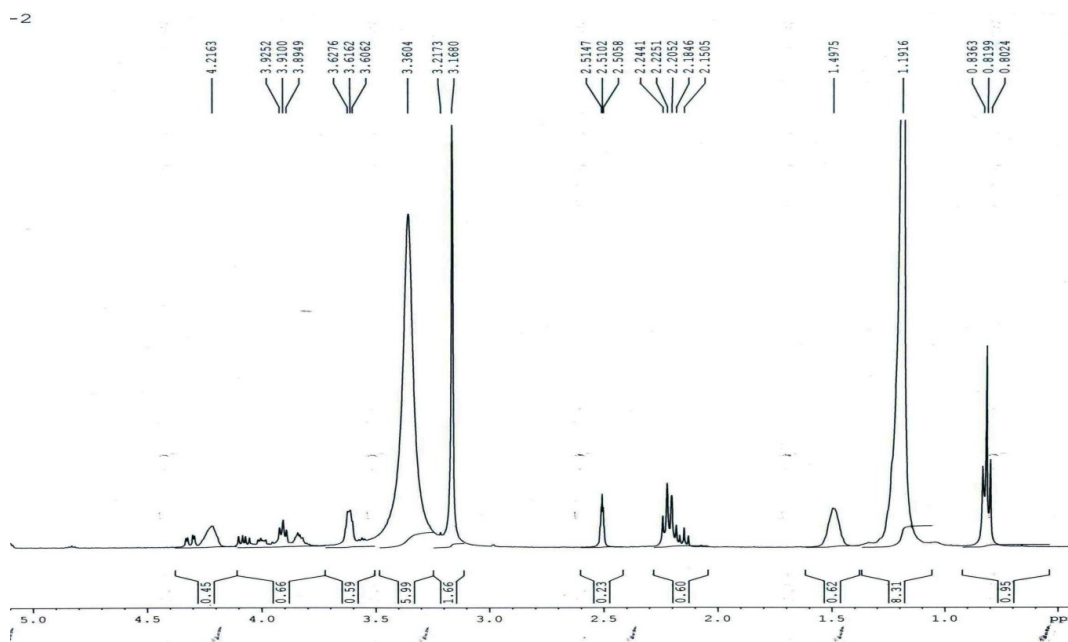


Figure 3e: NMR Spectroscopy of Quercetin-Phospholipid Complex (QCP)

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5. CONCLUSION:

Our study proves that TBE and TBEP have direct potential inhibitory action on breast cancer cells but the phytosome formulation show more antiproliferative activity as compared to pure extract. Hence it is anticipated that TBEP would prove to be a useful pharmaceutical formulation in treatment of breast cancer as compared to TBE. However further studies at molecular level have to be extended further for other cell lines also to identify specific mechanism that could induce growth inhibition. The most important to mention that this emerging pytosomes technology developed by Indena holds a lot of promises in the development of advanced herbal products.

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