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AN *IN VITRO* STUDY ON THE EFFECT OF *PISTACIA ATLANTICA* SUB *KURDICA* EXTRACT ON MICROTUBULE PROTEINS: A POTENTIAL ANTI-CANCER COMPOUND

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ABSTRACT: Natural products derived from plants and herbs have always had an important role in drug development. Especially in case of cancer, there is a huge demand for effective chemopreventive and chemotherapeutic agents with natural base and low toxic effects on normal non-cancerous cells. In this work, we have studied the effects of *Pistacia atlantica* sub *kurdica* (Baneh) extract on microtubule proteins and their biochemical features. Since microtubules are directly involved in spindle formation and cell division, they can be a very good target for anticancer drugs. We hypothesized that *Baneh* extract has the potential to inhibit microtubule polymerization and dynamics and as a result inhibit spindle formation during cell division. In order to analyze the effect of *Baneh* extract on microtubule proteins we decided to investigate these proteins biochemical characteristics in the presence of different concentrations of the extract. We used cell culture model to confirm cytotoxicity of *Baneh* extract. We then did some polymerization kinetics and dynamics study on microtubule proteins in the presence of various concentrations of the extract. Finally, we investigated the effect of *Baneh* extract on secondary and tertiary structural changes in tubulin protein. Our results indicate that *Baneh* extract an inhibit microtubule polymerization and dynamics, and can cause changes in the structure of tubulin protein. We had previously reported anticancer effects of *Baneh* extract on several cancer cell lines. Here, our observations further support the potentials of this natural compound as a promising candidate in novel chemo preventive drug development.

INTRODUCTION: Natural products derived from plants and herbs have always had an important role in drug development. From ancient times, plants and herbs were of huge interest for curing diseases and medicinal usages [1]. This is even more important in case of cancer research, where there is a huge demand for effective chemo preventive agents with natural base, that have high specificity for cancer cells and low toxic effects on other normal cells.

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Plant extracted chemical compounds are one of these natural occurring compounds and they have been of researchers' interests for many years now [2-7].

Pistacia atlantica sub *kurdica*, known locally as *Baneh*, is a plant from Anacardiaceae family; which grows in large populations in the western, central and eastern parts of Iran [8, 9]. *Baneh*'s nuts are used by natives as a snack and its gum is used in the production of chewing gum [10]. Several reports have mentioned the anticancer effects of *Baneh* as well as other family members of *Pistacia atlantica* family, in various cancer cell lines such as: prostate cancer, colon cancer, leukemia, lung carcinoma and breast cancer [10-15]. And all these strongly support the potentials of *Baneh* extract as an effective, natural, anticancer compound.

Previous studies on the effect of *Baneh* extract on cancer cells by our group have shown that this extract can induce apoptosis in cancer cell lines and it can also cease cell cycle progression [11, 12]. However, the effects of this extract on other cellular compartments are not well studied yet. Proper, continuous division is one of the crucial features of cancer cells. Microtubule proteins (MT) are one of the key role players in cell division, as they are directly involved in the formation of spindle and chromosome separation[16]. As a result, these proteins are considered as one of the important targets in cancer therapy [17, 18]. MT is a polymer of alpha and beta tubulin [19, 20]. MT polymerisation and depolymerisation is a dynamic process in cells and this dynamicity plays a crucial role in these proteins' normal function, including spindle formation and chromosome separation [16, 21].

MTs can interact with multiple proteins, the most important of them mainly known as Microtubule associated proteins (MAPs)- , and these interactions can affect various features of MTs such as polymer stability, length and dynamics [22, 23]. Many anti-cancer drugs such as colchicine and its derivatives, target MT proteins and they usually function by inhibiting chromosome separation upon cell division[18, 24, 25].

In this study, to further investigate the molecular mechanism of action of *Baneh* extract in protein level, we decided to look at the effect of this extract on MTs. We were wondering whether anticancer effects of *Baneh* extract could be partially because of its interaction with MT proteins and to investigate this hypothesis we did a series of biochemical studies on MT polymerization kinetics and dynamicity in the presence of *Baneh* extract. We also looked at the effect of *Baneh* extract on tubulin secondary structure.

MATERIAL AND METHODS:

Chemicals*:* All chemicals were obtained from Merck and Sigma Company. *Baneh* was freshly harvested from west of Iran (Kurdistan province) in July 2012.

Baneh extract preparation:

After identification and authentication of *Baneh* (*Pistacia atlantica* sub *kurdica*) in University of Tehran herbarium, the fruit was skinned, dried in darkness, powdered and stored at 4°C. This powder was extracted via percolation method by methanol: H_2O (70:30) for 3 days and was dried in air. The total extract was used for fractionation via liquid–liquid method by methanol then freeze dried, and stored until further use. Solubility of this sample was investigated in several solvents including water, chloroform and DMSO. Since the extract powder was not water soluble, DMSO was chosen as the solvent.

Identifying chemical content of Baneh extract:

In our previous work, we had reported the presence of polyphenols and flavonoids in *Baneh* extract [11, 12]. To study the terpenoids content in of the extract we used high performance liquid chromatography (HPLC). A C18 reverse-phase column (150 mm×3.9 mm inside diameter) was used with a4 µm Nova-Pack C18 cartridge (Water, Millford, MA). The mobile phase consisted of HPLC grade acetonitrile and was run isocratically at a flow rate of 1 ml/min. The injection volume was 20 µl and elution was monitored at 308 nm. According to terpenoid elution time, the peaks were collected and the Millennium workstation (version 3.05) chromatography manager was used to process the data (area integration, calculation, and plotting of chromatograms) throughout the method validation and sample analysis.

Cell culture:

Human neuroblastoma cell line, SK-N-MC- was obtained from Institute Pasteur cell bank. Cells were cultured in RPMI-1640 media containing 100 mg/ml penicillin and 100 mg/ml streptomycin, 15% Fetal Bovine Serum (FBS) and 5% horse serum. For cell viability assay, cells were cultured in 96-well plates for 24 hours. They were then treated with different concentrations of *Baneh* extract. In all experiments the final concentration of DMSO was adjusted to less than 1% in media. Cell viability was then tested using MTT assay, as previously described [26].

MT extraction and purification:

Tubulin was freshly purified from rat brain after two cycles of assembly and disassembly [27]. Rats were anesthetized by chloroform and brains were quickly extracted. Fresh brain was then

homogenized in a buffer comprising 0.1M piperazine-1,4-bis(2-ethanesulfonic acid), 1mM ethylene glycolbis (2-aminoethyl ether) tetraacetic acid, 2mM $MgCl_2$, and 1mM adenosine triphosphate (PEM buffer), and theresulting homogenate was centrifuged at 30,000g for30 minutes. The tubulin in the supernatant was polymerized at37˚ C for 45 minutes with 0.5 mM GTP and 33 % glycerol (v/v). Microtubules were pelleted by centrifugation (120,000g, 45 minutes, 4˚c), resuspended in PEM buffer, depolymerized at 4 C, and the mixture was centrifuged at 85,000g (4˚C)f or 45 minutes.

The supernatant was collected and the polymerization and depolymerization steps were repeated twice more. At the end, depolymerized fraction, containing tubulin dimers as well as MAPs was aliquoted and kept in -80 freezer until the experiment day. A portion of this fraction was subjected to anion exchange chromatography in a phosphocellulose column to remove residual MAPs for structural studies of tubulin protein. No impurities were observed after this step in Coomassie brilliant blue staining of10 % sodium dodecyl sulfate–polyacrylamide gel (**Fig 2a, d**).

MT polymerization kinetics analysis:

MT polymerization was recorded by monitoring the increasein turbidity at 350 nm and 37 °C in a Cary 100 spectrophotometer [28]. Depolymerization analysis was done by decreasing the temperature in site to 4 °C, and after at least 15 minutes, repolymerization was analyzed by increasing the temperature back to 37 °C.

Protein Fluorescence spectroscopy:

Intrinsic fluorescence of tubulin protein was measured in the absence and presence of different concentrations of *Baneh* extract. For all analysis, the concentration of purified tubulin was adjusted to 0.32 mg/ml (less than critical concentration to avoid tubulin polymerization). For *Baneh* treated proteins, samples were preincubated with the extract for 20 minutes on ice. Tryptophan excitation wavelength was applied at 295 nm and emission wavelength recorded, was adjusted to 300-450 with 5 nm bandwidth. We also investigated extrinsic dye fluorescence spectroscopy of tubulin proteinusing 8-Anilinonaphthalene-1-sulfonic acid (ANS) fluorometry. All samples were incubated with 50uM ANS for 7 minutes prior to recording fluo rescence. Excitation wavelength was adjusted to 380 nm and emission between 400-600 nm was recorded. All fluorescence experiments were done with a Cary Eclipse fluorescence spectrophotometer (Varian, Australia).

Circular Dichroism spectroscopy (CD):

Purified tubulin dimers were pre-incubated with various concentrations of *Baneh* extract for 20 minutes on ice. For all experiments, 0.32 mg/ml of tubulin protein was used. The secondary structural changes of protein were analyzed with a model 215 circulardichroism (CD) spectrophotometer (Aviv Biomedical, USA). The far-UV CD spectra were recorded from 190 to260 nm using a 1 mm path length quartz cuvette. Deconvolution and data analysis of CD spectra were performed using the program CDNN.

Transmission Electron Microscopy (TEM):

The polymerized microtubules in the presence of *Baneh* extract, as well as control chemicals (colchicine and DMSO) was diluted to 0.5 mg/ml in PEM buffer with 33 %glycerol at 37 C. 10 ul of samples were immediately placed on a Form var-coated electron microscopy grid, and were incubated at room temperature for 1 min. The liquid was subsequently wicked off with filter paper. 10 ul of 1% uranyl acetate was placed on the grid for negative staining and wicked off at room temperature after 1min. Sample grids were visualized using an HU-12A transmission electron microscope (Hitachi, Japan).

RESULTS:

Presence of two major types of terpenoids in *Baneh* extract:

HPLC analysis of Baneh extract indicates the presence of two major peaks at 308 nm (**Fig.1**). This is the wavelength at which terpenoids have high absorbance. The sharpness of the two peaks suggest that the extract consists of two major terpenoids with significant purity, and no other impurity is detected in the extract (**Fig.1**).

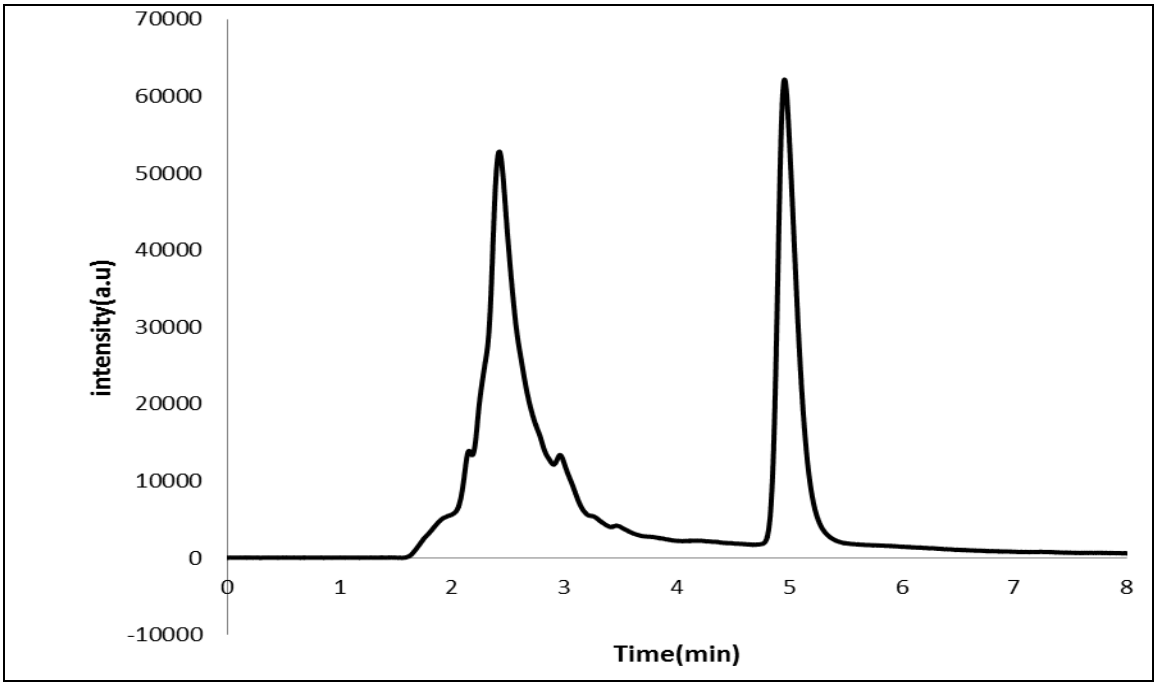

FIG.1: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) CURVE OF *BANEH* METHANOL EXTRACT. 20 µL OF EXTRACT WAS INJECTED AND ABSORBANCE WAS MONITORED AT 208 nm.

MT extraction, purification and polymerization assay:

a) **Verification of quality and activity of extracted microtubule proteins:**
To present the level of purity of extracted tubulin protein, we ran our samples on SDS-PAGE (**Fig 2a**). As it is shown in fig 2, after passing extracted MTs on anion exchange chromatography column, there is no impurity detected in the samples. Microtubule polymerization kinetics in purified samples was then analyzed in the absence and presence of colchicine- a known inhibitor of MT polymerization to make sure that it is the polymerization, not aggregation that is being measured(**Fig. 2b**). The capacity of MTs to depolymerize and repolymerize was measured as well (**Fig 2c**). As our results show, tubulin proteins show almost no drop in repolymerization ability after first depolymerisation, and this indicates high quality the samples.

b) **Polymerization kinetics in the presence of different concentrations of Baneh extract:** After verifying the activity and quality of extracted MT proteins, we analyzed the polymerization kinetics in the presence of different concentrations of *Baneh* extract (**Fig 3a**). Interestingly, the polymerization kinetics result indicate that *Baneh* extract has dual effects on MT polymerzaition kinetics. At low concentrations of the extract (0.025, 0.125 and 0.25 mg/ml), the nucleation phase is shortened. The curves also reach plateau phase after a longer logarithmic phase (OD number is higher) compared to the control groups. All these indicates enhanced polymerization in the presence of lower concentrations of *Baneh* extract. However, higher concentration of the same extract (0.0375 and 0.5 mg/ml) have an opposite effect of MT polymerization. The nucleation phase in 0.375 and 0.5mg/ml extract treated samples are longer than controls and the curves reach plateau phase after a short logarithmic phase (lower OD). And these all suggest the inhibition of polymerization in the presence of higher concentrations of *Baneh* extract. Another interesting point is that the slope of polymerization curve is the same in all groups and does not seem to change by alterations in *Baneh* extract concentrations.

c) Effect of Baneh extract on MT dynamicity: An important feature of MT proteins is their dynamicity and this can be manifested by the ability of tubulins to polymerize, depolymerize and repolymerize [21]. In order to see if *Baneh* extract can affect dynamicity in MTs, we tested the ability of *Baneh* extract treated MTs to depolymerize and repolymerize *in vitro*. According to our results, similar to its effects on polymerization kinetics, *Baneh* extract affects MT dynamics in a dual manner. At low concentrations (0.025, 0.125 and 0.25 mg/ml), once added the extract enhances polymerization and seems to have no inhibitory effect on depolymerisation and repolymerization. However, at higher concentrations (0.375 and 0.5 mg/ml), the extract seems to inhibit further polymerization, as well as the ability of MT to depolymerise and repolymerize. This effect is most noticeable at 0.5 mg/ml concentration of the extract (**Fig. 3**).

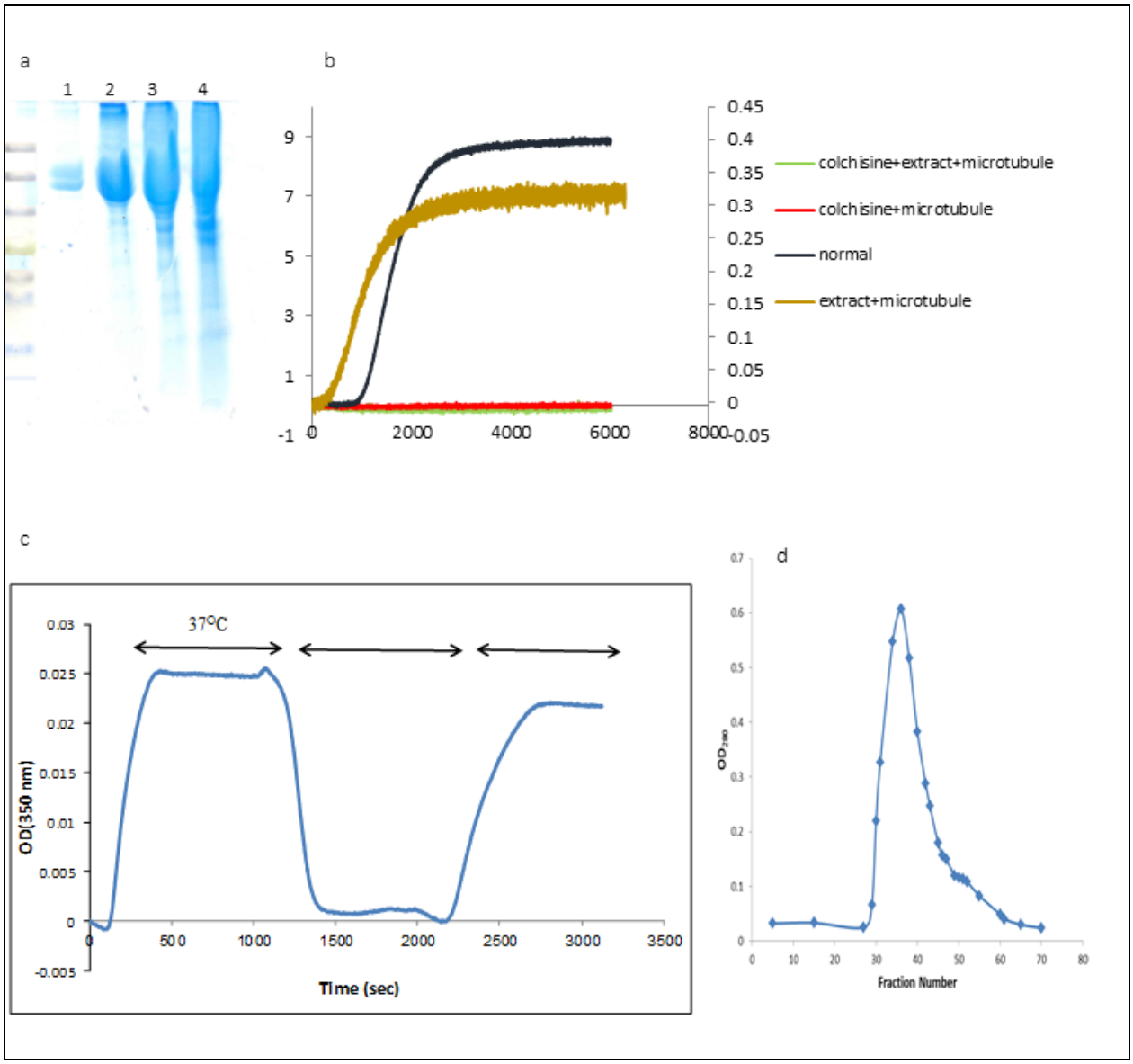

FIG. 2: a) SDS-PAGE OF DIFFERENT MICROTUBULE PURIFICATION STEPS: 1) FIRST DEPOLYMERISATION CYCLE, 2) FIRST POLYMERIZATION CYCLE, 3) LAST DEPOLYMERISATION CYCLE, 4) AFTER ANION EXCHANGE CHROMATOGRAPHY. b) POLYMERIZATION KINETICS OF MT IN THE PRESENCE OF COLCHICINE, EXTRACT OR BOTH. c) PURIFIED TUBULIN POLYMERIZATION AND DEPOLYMERISATION KINETICS. 4.58 MG/ML PURIFIED PROTEIN + 1MM GTP + PEM BUFFER. AFTER FIRST DEPOLYMERISATION, ONLY 12.3% OF ACTIVITY IS LOST IN SECOND REPOLYMERIZATION CURVE. d) FRACTIONS OF PROTEIN FROM ANION EXCHANGE CHROMATOGRAPHY, FRACTIONS 34-38 WERE COLLECTED AND USED FOR FURTHER EXPERIMENTS.

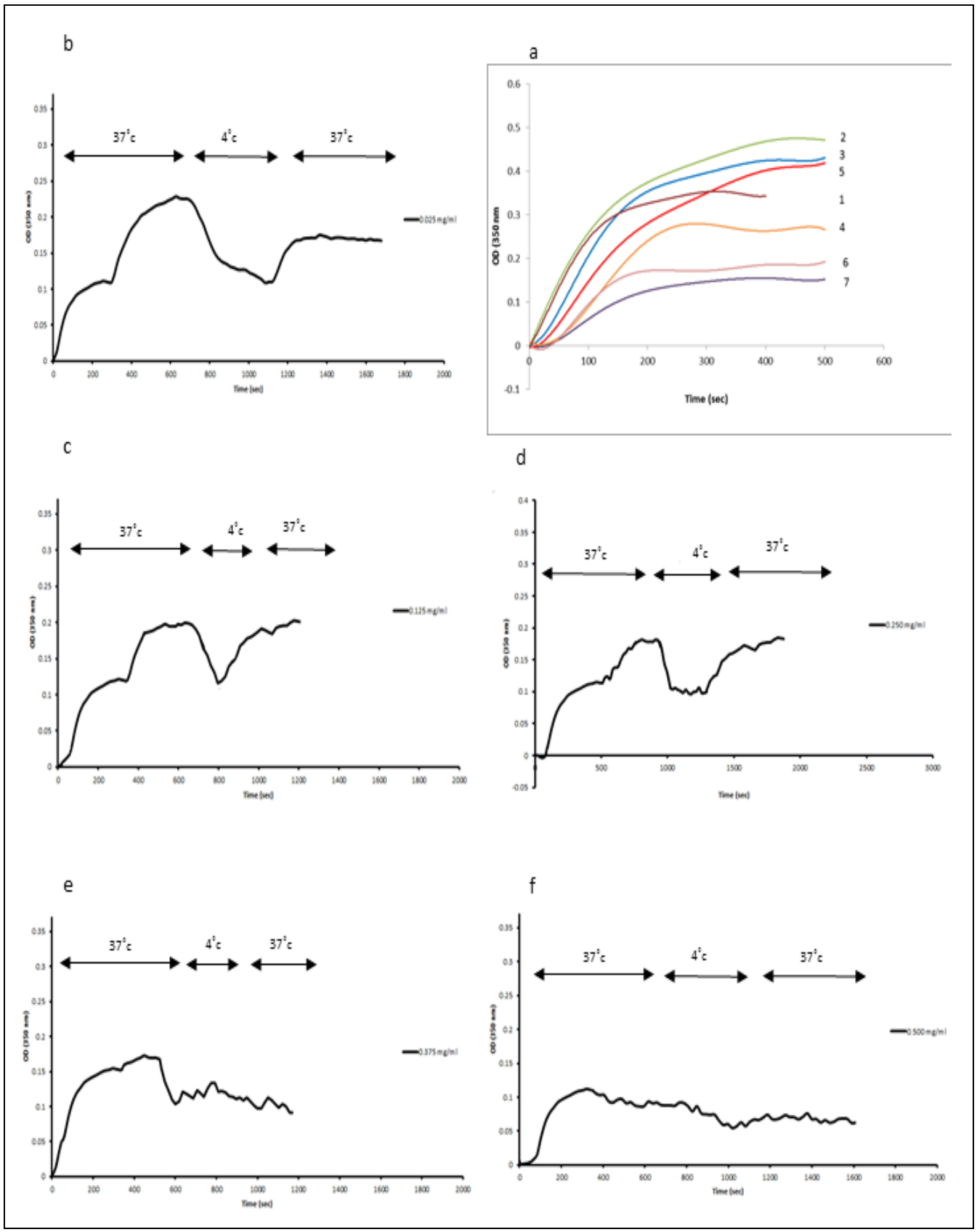

FIG. 3: A) MT POLYMERIZATION ASSAY IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF *BANEH* EXTRACT. 1) Mt + GTP, 2) MT + GTP + 0.025 mg/ml extract, 3) MT + GTP + 0.125 mg/ml extract, 4) Mt + GTP + DMSO, 5) Mt + GTP + 0.25 mg/ml extract, 6) MT + GTP + 0.375 extract and 7) MT + GTP + 0.5 mg/ml extract. b) MT polymerization and dynamics assay in the presence of different concentrations of *Baneh* extract, 0.025 mg/ml, c) 0.125 mg/ml, d) 0.25 mg/ml, e) 0.375 mg/ml and f) 0.5 mg/ml *Baneh* extract. . All protein concentrations were adjusted to 4.58 mg/ml, GTP concentration was 4 uM in all experiments and all polymerizations were done in PEM buffer.

Fluorescence spectroscopy of tubulin in the presence of different concentrations of *Baneh* extract: Intrinsic fluorescence spectroscopy results show a direct relationship between the concentration of *Baneh* extract and the intensity of intrinsic fluorescence signal (**Fig 4a**). As the concentration of extract increases, the maximum fluorescence signal decreases (**Fig 4b**). ANS Fluorescence spectroscopy results are also the same, showing a decrease in maximum fluorescence signal upon the increase in *Baneh* extract concentration. (**Fig 4c** and **d**).

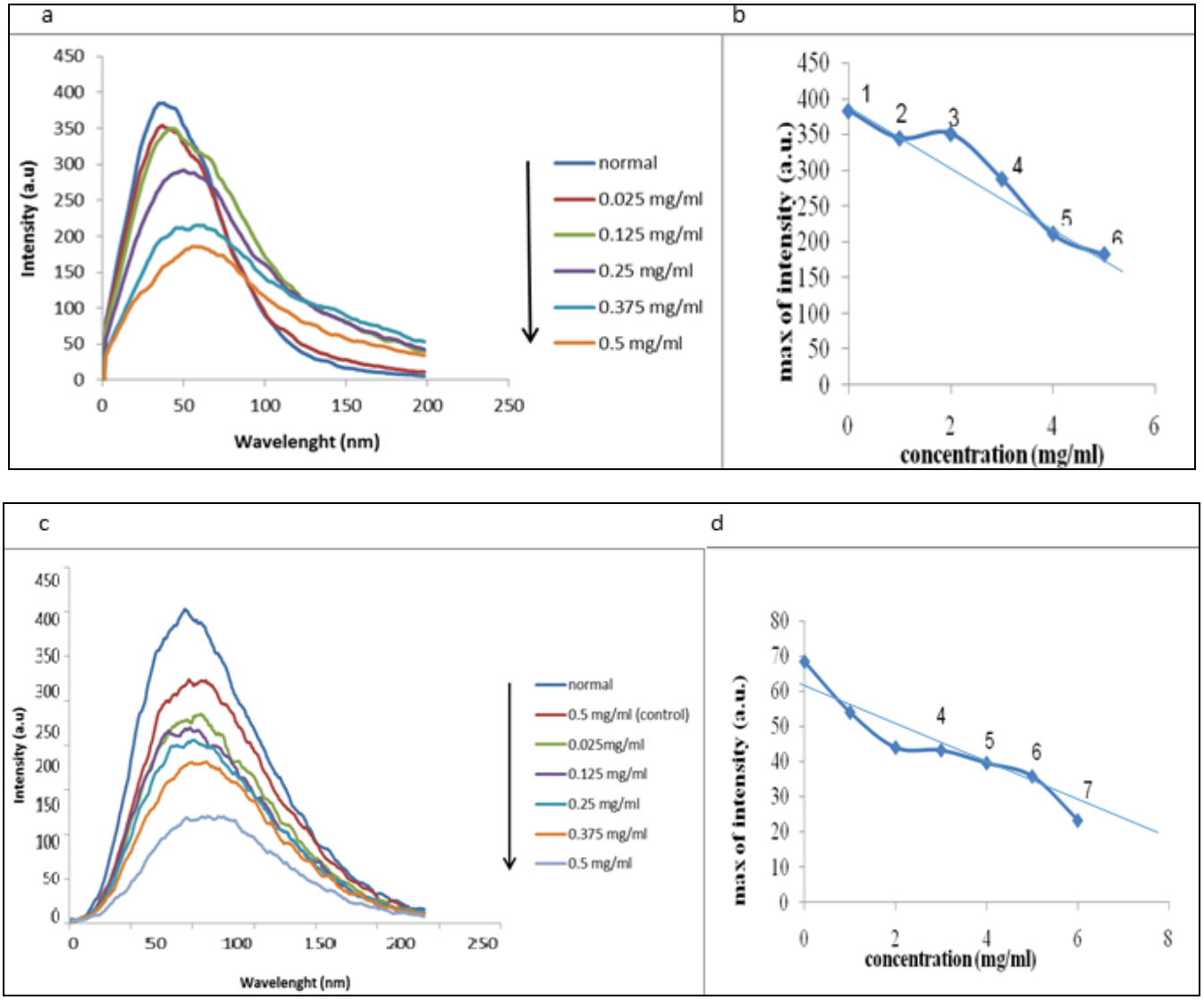

FIG.4: PROTEIN FLUORESCENCE SPECTROSCOPY: a) intrinsic protein fluorescence spectroscopy of tubulin in the presence of various concentrations of *Baneh* extract. 0.32 mg/ml tubulin was used for all samples. b) Maximum intensity of fluorescent signal curve. c) ANS fluorescence spectroscopy of tubulin protein in the presence of different concentrations of *Baneh* extract. 0.32 mg/ml purified tubulin and 10 uM ANS was used for all experiments. d) Maximum intensity curve of diagram c.

The effect of *Baneh* extract on tubulin secondary structure:

CD spectroscopy results show significant changes in secondary structure of the tubulin protein in the presence of *Baneh* extract. Our results show that incubation with *Baneh* extract can cause noticeable changes in the secondary structure of tubulin and these changes correlate with the concentration of *Baneh* extract (**Fig 5**). As **Table 1** shows, the secondary structure goes from a high β-sheet content in normal tubulin, towards a high α-helical content in *Baneh* extract-treated protein. The random coil content is also decreased in extract treated tubulin proteins. The effect is noticeable from 0.25 mg/ml and higher doses of extract treated tubulin.

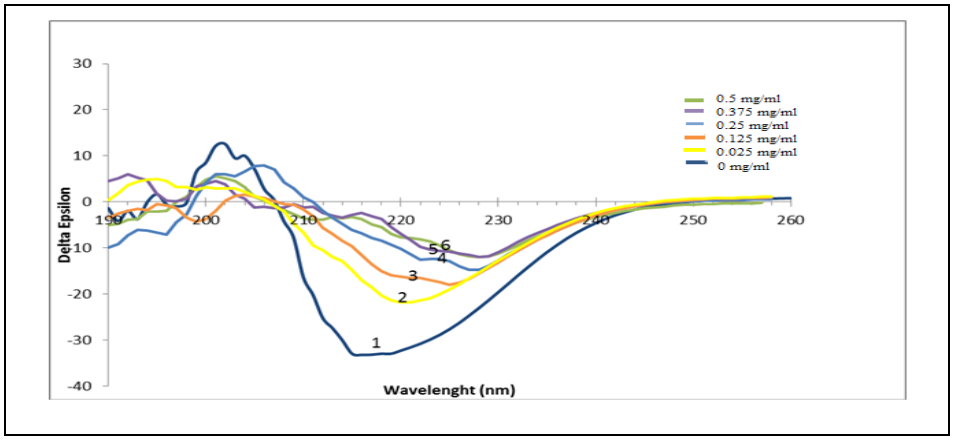

FIG.5: FAR-UV CD SPECTROSCOPY OF PURIFIED TUBULIN IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF *BANEH* EXTRACT. PROTEIN CONCENTRATION WAS ADJUSTED TO 0.32 MG/ML FOR ALL EXPERIMENTS.

TABLE 1: ALTERATIONS OF TUBULIN SECONDARY STRUCTURE IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF *BANEH* EXTRACT

Concentration of *Baneh* extract (mg/ml)	% α helix	% β sheet	% random coil	% β turns
0	6.3	48.8	36.4	19.3
0.025	6.3	48.8	36.4	19.3
0.125	6.3	48.9	36.4	19.3
0.25	60.1	9.9	22	8.7
0.375	61.6	11.3	18.8	9.6
0.5	61.6	11.3	16.2	7

TEM imaging of *Baneh* extract treated MTs: Incubation of tubulin with 0.25 and 0.5 mg/ml of *Baneh* extract eventually affects polymerization of MT. As figures(6d and e) show, by increasing the concentration of extract, less polymerized MT is observed and there are more unpolymerized protein in the environment (red arrows in fig 6).

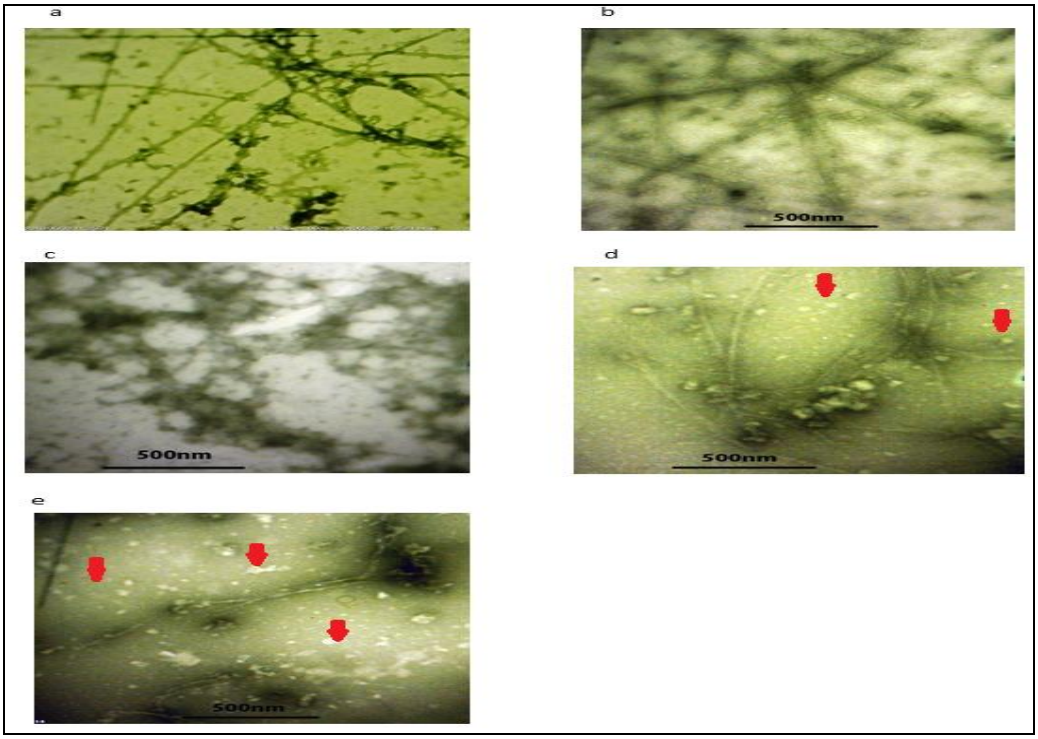

FIGURE 6: TEM IMAGES OBTAINED FROM POLYMERIZED TUBULIN IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF *BANEH* EXTRACT AND CONTROL CHEMICALS (COLCHICINE AND DMSO). a) Tubulin + 1mM GTP b) Tubulin + 1 mM GTP + DMSO, c) Tubulin + GTP + colchicine, d) Tubulin + GTP + 0.25 mg/ml extract and e) Tubulin + GTP + 0.5 mg/ml extract. 50,000x resolution, for all samples, 4.5 mg/ml purified protein and 4uM GTP was incubated for 45 minutes in PEM buffer.

Cytotoxicity of *Baneh* extract in a neuroblastoma cell line: In order to observe the effect of different concentrations of *Baneh* extract on cells, we did a cell viability assay on a human neuroblastoma cell line. As the results indicate (**Fig. 7**), 0.3 mg/ml and higher concentrations of the extract have cytotoxic effects on cells and cause a significant decrease in cell viability.

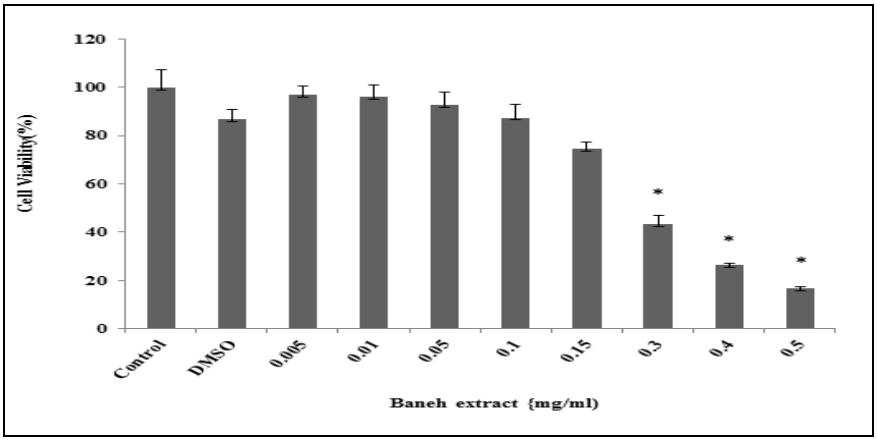

FIG.7: CELL VIABILITY ASSAY FOR SK-N-MC CELL LINE INCUBATED WITH VARIOUS CONCENTRATIONS OF *BANEH* EXTRACT. THE MAXIMUM DMSO CONCENTRATION WAS LESS THAN 1% IN ALL SAMPLES AND THE CONTROL HAS THE SAME AMOUNT OF DMSO AS THE MAXIMUM DMSO USED IN INCUBATIONS. INCUBATION PERIOD WAS 24 HOURS FOR ALL EXPERIMENTS.

DISCUSSION: Natural compounds and plant derived substances have always been of great interest for drug development [1, 3, 7, 29]. The anti-cancer effects of plant derived chemicals such as flavonoids, terpenoids, proanthocyanidins and phytoesterogens have been vastly investigated [29-33]. And as a result, such natural compoundsare believed to be good candidates for novel anti-cancer drug development. MT network can be a very good target of anti-cancer drugs, since these proteins play a crucial role in all eukaryotic cells and their ability to polymerize and form protein tubules with various lengths, as well as the dynamics of the MT network is very important for cell viability, division and function[16, 18, 24].

Previous reports from several labs including our group, had mentioned anti-cancer effects of *Baneh* extract on several cancer cell lines [11, 12, 14, 15, 17]. However, the effect of *Baneh* extract in protein level is not well studied. As a result, we decided to check the effects of this extract on biochemical features of MT proteins.

Our results strongly suggest that chemicals present in *Baneh* extract have noticeable effects on microtubule polymerization kinetics as well as its dynamics. Surprisingly at low concentrations, polymerization is enhanced by the presence of the extract and its dynamics is not much changed. At higher doses, however, *Baneh* extract seems to affect both polymerization ability and dynamics in a negative way and it inhibits microtubule formation. Our structural analysis also suggest huge changes in secondary (α-helical and β-sheet content of tubulin), as well as tertiary structure (Intrinsic and ANS fluorometry) of tubulin. As fig 4 shows, there is a noticeable change in protein fluorescence in the presence of extract and this change directly correlates with changes in concentration of the *Baneh* extract. As the concentration increases, the maximum peak of fluorescence emission is reduced, and the fluorescence peak position also moves toward higher wavelengths, meaning that there is a change of position for tryptophan residues in protein.

Moreover, as our ANS fluorometry results show, by increasing the concentration of *Baneh*extract, fluoresce emission is reduced. Tubulin protein has one high affinity and a couple of low affinity binding sites for ANS. Changes in ANS fluorescence intensity suggests that in the presence of high concentrations of *Baneh* extract, there is a structural change occurring in tubulin protein in a manner that makes the ANS binding sites (which are hydrophobic regions) inaccessible for interacting with this molecule anymore. All these results strongly suggest that high concentrations of *Baneh* extract can cause structural changes in

tubulin and this might be a way the extract inhibits MT polymerization. Our TEM imaging results also show that at high concentrations of *Baneh* extract, (0.375 and 0.5 mg/ml), the number of polymerized MTs is less than control groups and the presence of unpolymerized chunks of protein in environment suggests that tubulin dimers cannot successfully interact with each other to form long, stable microtubules. Finally, our cell viability assay results show that the high dose amounts of *Baneh* extract decreases cell viability significantly.

MT polymerization depends on various factors: free tubulin concentration, GTPase activity of tubulin protein and presence of several MAPs (*in vivo*) can all affect MT polymerization[21]. Changes in any of these factors can then affect the ability of tubulin protein to form active and dynamic polymers. We have used purified tubulin for our polymerization assay as well as our structural analysis experiments and for that reason it is safe to conclude that *Baneh* extract is directly interacting with tubulin proteins and the effects we have observed in this study is on tubulin protein itself rather than MAPs. However the mechanism through which the extract is inhibiting MT polymerization, needs to be further analyzed. Our explanation regarding our observations is that *Baneh* extract induces such structural changes in tubulin protein that results in its reduced ability to self-interact and polymerize. However further investigations need to be performed in order to firmly confirm this matter.

C**ONCLUSION AND FUTURE DIRECTION:**

In conclusion, our results suggest that *Baneh* extract seems to be a promising candidate for further analysis in search of a natural, plant-derived anti-cancer compound. For now, our HPLC results suggest that there are two pretty pure terpenoid-based compounds present in our extract. Future direction will include identifying the exact compounds present in the extract and studying their effects on MT proteins individually.

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