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Development and Evaluation of Polyherbal Anticancer Tablets: A Review

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Abstract: *Cancer is a malignant abnormal growth of cells, one of the most dreaded and complex diseases involving numerous tempo spatial changes in cell physiology, which ultimately lead to malignant tumours, Neoplasia. More than 100 types of cancers have been reported, symptoms vary depending on the type and treatment may include chemotherapy, radiation, and/or surgery. Herbal remedies are assumed to be safe, cause less complications and are less likely to cause dependency. The anticancer activity of medicinal plants is due to antioxidant compositions. Therefore, the various standardized combinations and preparation of dose and dosage regimen of the active components assessed for their synergistic effects, which could play a critical role in cancer treatment. Evaluation parameters to assess the in vitro anticancer activity includes Caspase-3, Caspase-9, Chromosomal Aberration Assay, Alamar Blue Resazurin Reduction assay, LDH assay, XTT assay, Sulforhodamine-B assay, MTT assay, DNA Fragmentation assay, ELISA assay, Neutral Red Uptake Cytotoxic assay, SRB assay, Tryphan Blue assay. Evaluation of dried extract or granules includes Bulk density, Tapped density, Carr's index, Hausner's ratio, Angle of repose while the tablets evaluated by Drug-Excipient Compatibility Study by FT-IR, Stability studies, Hardness, Thickness, Weight Variation, Friability, Disintegration Time, In vitro Dissolution test*

Keywords: *Cancer*.

I. INTRODUCTION

Cancer persists to characterize the largest cause of mortality in the humanity and claims over 6 million lives every year [1]. An enormously potential strategy for cancer prevention today is chemotherapy, which is defined as the use of synthetic or natural agents used alone or in combinations to block the expansion of cancer in humans. Plants, vegetables and herbs used in the folk and traditional medicine have been accepted currently as one of the chief source of cancer chemoprevention drug discovery and development [2].

Medicinal plants have been used as remedies for human diseases for centuries. The reason for using them as medicine lies in the fact that they contain chemical components of therapeutic importance [3]. The medicinal significance of plants lies in some chemical substances usually secondary metabolites, that bring into being a definite physiological action on the human body. The most significant bioactive compounds are alkaloids, flavanoids, tannins and phenols [4].

The investigation for anticancer agents from plant sources started in the 1950s and consequences in the detection and development of the vinca alkaloids, vincristine, and the isolation of the cytotoxic podophyllotoxins and so far [5-6]. More than 60% of presently used anticancer agents are derived in one way or another from natural sources [7-8]. Some of the medicinal plants like *Cinnamomum tamala, Madhuca longifolia, Adina cordifolia, Sida Veronicaefolia, Terminalia arjuna, Catharanthus roseus, Zingiber Officinalis, Alium cepa, Aloe barbadensis, Citrus medica, Nicotiana tabacum, Allium sativam, Emblica officinalis, Glucyrrhiza glabra, Ocimum sanctum, Curcuma longa* etc., evaluated and showed prominant anticancer activity. Biological dynamic components from plants are significant and imperative source of new drugs that are likely to lead to advancement in treatments of various kind cancers. As chemotherapy also destroys the normal cells along with cancer cells, sometimes cancer cells can develop resistance to treatment through mutations

[9].Therefore, screening for the abundance of biodiverse components is important for research before the forests are lost to deforestation.

Herbal formulations are the formulation prepared by plant extracts either of bark, leaves, stem or roots or whole plant material with suitable excipients. These extracts contain some of the active constituents, thus with suitable excipients or base different formulations are prepared and standardized with suitable methods. When two or more herbs are used in formulations, they are known as polyherbal formulations.

It is strongly recommended to bring out more cellular and molecular studies to clear mechanisms in anticancer actions of herbs. A great number of *in vitro* and *in vivo* methods have been developed to measure the efficiency of natural anticancer compounds either as pure compounds or as plant extracts. *In vitro* methods like, tryphan blue dye exclusion assay, LDH (Lactic dehydrogenase) assay, MTT assay, XTT assay and Sulforhodamine B assay and many more are most commonly used for estimating anticancer properties of natural products from medicinal plants. Dried extract evaluation include determination of Bulk density, Tapped density, Carr's index, Hausner's ratio, Angle of repose while the tablets evaluated by Drug-Excipient Compatibility Study by FT-IR, Stability studies, Hardness, Thickness, Weight Variation, Friability, Disintegration Time, *In vitro* Dissolution test.

II. EVALUATION PARAMETERS

2.1 In- vitro Evaluation Parameters

2.1.1 Caspase-3

Caspases (Cysteine-requiring aspartate proteases) are a family of proteases that mediate cell death. It is also important to the process of apoptosis. Caspase-3 is a member of the CED-3 subfamily of caspase and is one of the critical enzymes of apoptosis. Caspase-3 processes procaspase 2, 6, 7 and 9 and specifically cleaves most of caspase- related substrates. Many key proteins including nuclear enzyme activate poly (ADP-ribose), and polymerase (PARP)[10], the inhibitor of caspase-activated deoxyribonuclase (ICAD) gelsolin and fodrin, which are the proteins, involved in the apoptosis regulation[11]. This cleavage is part of the mechanism leading to cell death. Caspase-3 plays a central role in mediating nuclear apoptosis including chromatin condensation and DNA fragmentation as well as cell blebbing. The activity of caspase- 3 in cells and tissues of the body is either a marker or indicator of cell death[12].

Caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (AC-Devo-PNA) by Caspase-3, resulting in the release of the p-nitroanilide (PNA) moiety. The p-nitroanilide has a high absorbance at 405 nm (ΣmM = 10.5). The concentration of PNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined PNA solutions.

Caspase-3

Caspase-3 is a crucial component of the apoptotic machinery in many cell types. The activation of caspase-3 is a central event in the process of apoptosis[13,14].

Caspase-9

Caspase-9, also known as ICE –Lap6, Mch6, is an upstream proenzyme in the cascade of enzymatic reactions required to induce cellular apoptosis[15]. During the release of mitochondrial cytochrome-c, and caspase-9 activated following their association with the protein complex of Apaf and cytochrome-c. The active caspase-9 is generated through protein phosphoylation events and the optimal cleavage recognition sequence for caspase-9 is Leu-Glu-His-Asp-7 (LEHD). The caspase-9 is associated with both pro-apoptotic and anti-apoptotic proteins as well as its regulatory function during embryonic development. This suggests that caspase-9 is a key regulatory enzyme of apoptosis [16-17]. The Caspase-Glo® 9 assay is a homogenous luminescent assay that measures caspase-9 activity. Addition of a single Caspase-Glo®-9 reagents in an add-mix measure formant resulting in cell lysis. This is followed by caspase cleavage of the substrate and the generation of a glow type luminescent signal produced by the luciferase reaction. The signal generated is proportional

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to the amount of caspase activity present. Figure 1 show the process involved in the measurement of caspase-9 in cells and tissues.

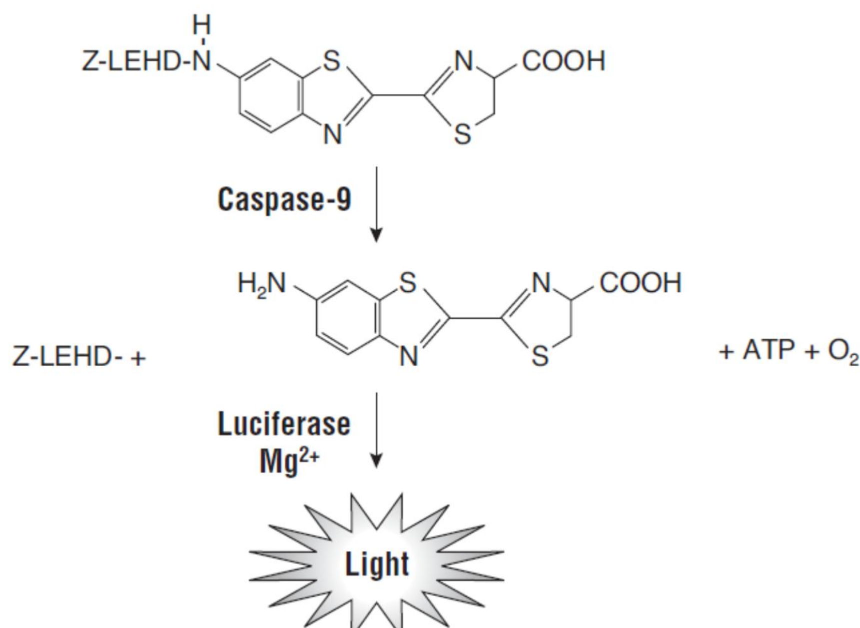

Figure 1: Caspase-9 cleavage of the luminogenic substrate containing the LEHD sequence.

2.1.3 Chromosomal Aberration Assay

For chromosomal assay, different doses of *Aloe vera extract* i.e. 250, 500 and 750mg/kg body weight were administered. *Aloe vera* extract were dissolved in double distilled water and administered as single dose in 0.2 ml per mouse i.p. to 6 animals, 24 hours before the treatment of cyclophosphamide. Control mice were administered an equal volume of vehicle alone. The positive control group also received a single i. p. injection of 50 mg/kg CP in 0.9% saline. The animals were sacrificed by cervical dislocations and bone marrow cells were harvested. Colchicine (4 mg/kg b. wt.) was administered intraperitoneally 2 hrs. before the harvest of the cells. The slides prepared essentially as per modified method of Preston *et al* (1987) [18]. Briefly, femur bones were excised and the bone marrow extracted in 0.56% KCl. The harvested cells were incubated at 37°C for 20 minutes. and then centrifuged for 10 mins. at 1000 rpm. Cells were fixed in Carnoy's fixative (methanol: acetic acid = 3:1) and bursed opened on clean slides to release chromosome. The slides were stained with 5% Giemsa solution for 15 mins and then put in xylene and mounted with DPX. A total of 100 well spread metaphase plates were scored for chromosomal aberrations at a magnification of 1000 X (100 X 10) for each group. Different types of chromosomal aberration such as chromatid breaks, gaps, centromeric association, etc. were scored and expressed as % of chromosomal aberrations. The statistical significance was determined using Student's 't' test [18].

2.1.4. Alamar Blue Resazurin Reduction assay

"Alamar Blue" resazurin reduction assays were conducted as described [19]**.** Cell suspended in 100 µl of DMEM were seeded in 96-well plates at a density of 5 × 103 cells per well and incubated for 24 h. All extracts were serially diluted into supple-mented media using a separate 96-well plate, applied to the cells, and incubated for 48 h. Following the incubation, 100 µl of fresh media, (containing 10% (v/v) of a 860 µM solution of resazurin in PBS) was added to the cells, and incubated for 2 to 4 h. The fluorescence intensity of the dye was then quantified by a SpectraMax M5 plate reader using excitation at 560 nm. IC50 values were calculated from the fluorescence intensity values, by using an exponential decay curve fit. DMSO was used as a negative control, whereas Nile Blue A [20] was used as a positive control.

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2.1.5. LDH assay

Lactic dehydrogenase activity is spectrophotometrically measured in the culture medium and in the cellular lysates at 340 nm by analyzing NADH reduction during the pyruvatelactate transformation. Cells are lysed with 50 mM Tris-HCl buffer, pH 7.4 + 20 mM EDTA + 0.5 % Sodium Dodecyl Sulfate (SDS), further disrupted by sonication and centrifuged at 13,000 X g for 15 min. The assay mixture (1ml final volume) for the enzymatic analysis consists of 33 µl of sample in 48 mM PBS, pH 7.5 + 1 mM pyruvate and 0.2 mM NADH. The percentage of LDH released is calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium [21].

2.1.6. XTT assay

(2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]- 2Htetrazolium- 5-carboxyanilide inner salt (XTT) assay is used. The tetrazolium salt, XTT, is especially useful in quantifying viable cells. This assay is designed for the spectrophotometric quantification of cell growth and viability without the use of radioactive isotopes and is based on the cleavage of yellow tetrazolium salt, XTT, to form an orange formazan dye by metabolically active cells. XTT cleavages into an orange formazan dye by the mitochondrial enzyme, dehydrogenase, occurs exclusively in living cells. Cells are grown in growth medium plus 10 % FBS in 96-well plates until 70-80 % confluence. They are then treated with the appropriate drug sample for 24 h. An XTT assay is performed at the end of incubation. Briefly, 50 mL of XTT labeling mixture solution is add to each well, and the cells are incubated at 37 °C for 4 h. The formazan dye formed is soluble in aqueous solutions and the optical density at 450 nm is compared with that of control wells with a screening multiwell spectrophotometer enzymelinked immunosorbent assay (ELISA) reader. The reference wavelength is 650 nm.[22].

2.1.7. Sulforhodamine-B assay

Sulforhodamine B assay is a bright pink aminoxanthene dye that binds to basic amino acids in mild acidic conditions and dissociates under basic conditions. Cells are plated in 96-well flat bottom plates at 5000-10000 cell/well. The difference in cell numbers plated adjusts for differences in the growth rates of the various cell lines. Cells are allowed to adhere to the wells overnight, then the samples are added to triplicate wells in serial 3-fold dilutions. Water is added to the control wells at a 1:10 dilution in medium. These plates are incubated at 37 °C, 5 % CO2 for 3 days, then assayed for growth inhibition using a sulforhodamine B (SRB) assay. The cells are fixed by the addition of cold 50 % trichloroacetic acid to a final concentration of 10 %. After 1 h incubation at 4 °C, the cells are washed five times with deionized water. The cells are then stained with 0.4 % SRB (Sigma) dissolved in 1 % acetic acid for 15-30 min and subsequently washed five times with 1 % acetic acid to remove unbound stain. After the plates are air dried at room temperature, the bound dye is solubilized with 10 mm Tris base and the plates are analysed on a microplate reader (Molecular Devices) at 595 nm [23].

The % growth inhibition is calculated as $= \dfrac{\text{Control sample}}{\text{Control}} \times 100$

2.1.8. MTT assay

The MTT assay, based on the conversion of the yellow tetrazolium salt-MTT, to purple-formazan crystals by metabolically active cells, provides a quantitative determination of viable cells. Cells are plated on to 96 well plates at a cell density of 2×105 mL-1 per well in 100 µL of RPMI 1640 and allowed to grow in CO2 incubator for 24 h (37 ˚C, 5 % CO2). The medium is then removed and replaced by fresh medium containing different concentrations of sample for 48 h. The cells are incubated for 24-48 h (37 ˚C, 5 % CO2). Then, 20 µL MTT ([3- (4, 5-dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide]) stock solution (5mg/mL in PBS) is added to each well and incubated for 5 h. The medium is removed and 200 µL DMSO is added to each well to dissolve the MTT metabolic product. Then the plate is shaken at 150 rpm for 5 min and the optical density is measured at 560nm. Untreated cells (basal) are used as a control of viability (100 %) and the results are expressed as % viability (log) relative to the control [24].

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2.1.9. DNA Fragmentation assay

HeLa cells (3 x 106 /ml) were seeded into 60mm Petri dishes and incubated at 37°C with 5% CO2 atmosphere for 24 h. The cells were washed with medium and were treated with extract, standard drug and incubated at 37ºC, 5% CO2 for 24 hrs. At the incubation time ended, the chromosomal DNA of cancer cells was prepared with G Biosciences, USA apoptotic DNA ladder kit. The recovered DNA was loaded onto 2% agarose gel electrophoresis and run 50 V/cm for 3 hrs. The gel was visualized under UV transilluminator and photographed [25].

2.1.10. Neutral Red Uptake Cytotoxic assay

NR dye (3.3gm) was dissolved in 100 ml of double distilled water and then this stock solution was filtered by using syringe filter. It was stored at room temperature and used within 6 months. 1 ml of NR stock solution was dissolved in the 99 ml of culture media to get the final concentration 0.33%. Incubated cell plates were taken out from the incubator, and discard the culture media from the plates. Culture media was replaced by the extract containing culture media. Then the plates were incubated in CO2 incubator for 24 hrs for the action of extracts. The extract containing culture media was then replaced with NR-containing medium. Plates were again placed to incubator for 4-8 hours depending on cell type and maximum cell density. At the end of the incubation period, the medium was carefully removed and the cells were quickly washed with PBS. The washed solution was removed and the incorporated dye was then solubilized in a volume of Neutral Red Assay Solubilization Solution (ethanolic acetic acid) equal to the original volume of culture medium. The plates were allowed to stand for 10 minutes at room temperature. Gentle stirring in a gyratory shaker or pipetting up and down (trituration) enhanced mixing of the solubilized dye. The background absorbance was measured at 540 nm using ELISA reader to get optical density and pictures were captured using microscope [26]. Then calculate the % inhibition using the formula

% inhibition = [(OD of untreated)-(OD of drug Treated) /(OD of untreated)]*100

2.1.11. SRB assay

On addition of the drug, the plates were incubated further for 48 hours at 37oC in humidified CO2 (5%) incubator. After incubation, 50μL of 30% TCA was added to fix the cells to the bottom of the wells. After 60 minutes incubation at 4°C, plates was washed gently under tap water and air dried at room temperature. Then 100 μL SRB (sulforhodamine B) reagents was added into each well and left for 15minutes and the SRB dye was removed by washing the plates with tap water. 1% acetic acid was used to remove unbound SRB dye. After air drying, 0.1ml of 10mM UN buffered TRIS base was added and the absorbance was read on the Elisa plate reader at the wavelength of 540nm with reference to 690nm. Optical density of drug treated cells was compared with that of control cells and growth inhibition as calculated as percent values [27, 28].

2.1.12. Tryphan Blue assay

The trypan blue dye exclusion assay is the most commonly utilized test for cell viability. In this assay, the cells are washed with HBSS (Hank's Buffered Salt Solution) and centrifuged for 10 -15 min at 10,000 rpm. The procedure is repeated thrice. The cells are suspended in known quantity of HBSS and the cell count is adjusted to 2 x 106 cells /ml. The cell suspension is distributed into Eppendorf tubes (0.1 ml containing 2 lakhs cells). The cells are exposed to drug dilutions and incubated at 37 °C for 3 h. After 3 h, dye exclusion test, that is, equal quality of the drug treated cells are mixed with tryphan blue (0.4 %) and left for 1 min. It is then loaded in a haemocytometer and viable and non-viable count are recorded within 2 min. Viable cells do not take up colour, whereas dead cells take up colour. However, if kept longer, live cells also generate and take up colour [29]. The percentage of growth inhibition is calculated using the following formula:

$$\text{Growth inhibition (\%)} = 100 - \frac{\text{(Total cells-Dead cells)}}{\text{Total cells}} \times 100$$

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2.2. Evaluation of dried extract or granules

2.2.1. Bulk density (D_B)

It is determined by measuring the volume of the known mass of powder that has been passed through a screen into a measuring cylinder [30].

Graduated Cylinder method- Powdered sample (M), previously passed through sieve no. 18 to break up agglomerates that may have been formed during storage, is introduced into a 100ml graduated cylinder without compacting. The apparent volume (Vb) is then read to the nearest unit. The bulk density is calculated in gm/cm^3 by formula.

$$D_B = M/Vb$$

Where, M = weight of the sample and VB =mean bulk volume of the sample.

2.2.2. Tapped density (D_T)

Specific quantity of the screened sample was poured at an angle of about 45° into a clean dry 100 mL measuring cylinder, placed on a Stampfvolumeter and tapped until no further change in volume occurred [30]. The constant volume was noted.

$$D_T = M/V_T$$

where M = weight of the sample and V_T =tapped volume of the sample.

2.2.3. Carr's index

The Compressibility index (Carr's index) is a measure of the propensity of a powder to be compressed. It is determined from the bulk and tapped densities. In theory, the less compressible a material the more flowable it is. As such, it is measures of the relative importance of interparticulate interactions. In a free flowing powder, such interactions are generally less significant, and the bulk and tapped densities will be closer in value. For poorer flowing materials, there are frequently greater inter-particle interactions, and a greater difference between the bulk and tapped densities will be observed [31, 32]. These differences are reflected in the Carr's Index which is calculated using the following formulas:

Comperessibility index = $\{(DT - DB)/DT\} \times 100\ \%$

Where, D_B = Bulk Density, D_T = Tapped Density

2.2.4. Hausner's ratio (HR) Compressibility index (CI)[33]

The Hausner ratio (HR) and Compressibility index (CI) of the samples were derived from Eqs. HR = DT/DB

2.2.5. Angle of repose and Flow Rate

The fixed-height cone method was used. A glass funnel was fixed at a 10 cm height over a flat horizontal surface. The exit/orifice of the funnel was temporarily blocked with a flat ruler. A clean white paper was spread beneath the funnel on the horizontal surface for collection of the powder. Specific quantity of the sample was then weighed and poured into the funnel. The exit of the funnel was then sharply unblocked and the time (seconds) taken for the sample to completely flow out onto the horizontal surface was measured with a stop clock. The height and radius of the cone formed were measured with a ruler[34]. Flow rate and angle of repose of the sample were calculated as in Eqs

Flow rate = M/t

Angle of repose = $\tan A = h/r$

where M = mass (g) of the sample, t = time (seconds) taken for the sample to completely flow out of the funnel, A = angle (o) formed by the sample cone, and h = height (cm) of the sample cone formed after flowing from the funnel.

2.3. Evaluation of tablets

2.3.1. Drug-Excipients Compatibility Study by FT-IR

Compatibility of the drug with excipients was determined by FT-IR spectral analysis, this study was carried out to detect any changes on chemical constitution of the drug after combining it with the excipients. The samples were taken for FT-IR study. IR spectra of drug in KBr pellets at moderate scanning speed between 4000-400 cm-1 was carried out using

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FTIR. The peak values (wave number) and the possibility of functional group are shown in spectra which compare with standard value. The comparison of these results with chemical structure shows that the sample was pure [35].

2.3.2. Stability studies

The optimized formulation of the drug was subjected to accelerated stability studies at specified conditions of temperature and relative humidity of 25^0C/60% RH, 30^0C/60% RH and 40^0C/75% RH for 3 months [36].

2.3.3. Hardness and friability

Hardness was determined by using a Monsanto tablet hardness tester (n = 6).
Friability of pre-weighed ten tablets was determined by using Roche friabilitor at 25rpm for 4 min. The tablets were dedusted and reweighed.

%Friability = (Loss in weight/Initial weight) x 100

2.3.4. Thickness

The tablet thickness was calculated by Vernier calipers. Tablet was put in between two jaws vertically and measured thickness and 6 tablets were used for this test and expressed in mm.

2.3.5. Weight Variation

The tablets were evaluated as per I.P., 1996 for weight variation (n = 20) using 1mg sensitivity balance.

2.3.6. Disintegration Time

Disintegration test was performed using a USP disintegration apparatus, with 900ml distilled water at 37±2°C. Time required for complete disintegration of six tablets was recorded.

2.3.7. *In vitro* Dissolution test

The release of tablets was determined using USP dissolution test apparatus. Dissolution was examined in 900ml of distilled water with two tablets placed in each of 6 dissolution vessels. The temperature was maintained at 37 ± 0.2°C. Samples each of 10 ml were withdrawn at 10, 20, 30, 40, 50 and 60 min time interval, filtered through wattman filter paper and replaced with an equal amount of fresh dissolution medium. Equal volumes of the filtered specimens withdrawn were combined and used as the pooled sample, as test solution. The % release per tablet was determined [37].

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