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# THE IMPACT OF HERBAL INFUSION KAHWAH ON OXIDATIVE STRESS AND BREAST CANCER IN MOUSE MESENCHYMAL STEM CELL LINE C3H10T1/2 AND MCF-7

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**Abstract**: Obese people are more likely to develop triple-negative breast cancer (TNBC), which is linked to a condition of chronic low-grade inflammation. Epidemiological studies, on the other hand, showed that Increased consumption of polyphenol-rich fruits and vegetables appears to play a crucial role in lowering the prevalence of certain cancers. In addition to natural products, Kahwah (a mixture of green tea and spices) have many desirable properties so that they can be used for medicinal purposes. The Kahwa is an exotic blend of Kashmiri green tea leaves, whole spices, luscious saffron, and nuts steeped in a special kettle known as a samovar. Saffron is high in vitamin B12 and antioxidants, which help with immunity, stress relief, and digestion. Saffron, cardamom, cinnamon, clove, and green tea leaves Spices" effects on various types of stem cells have been studied in several studies Dietary-derived polyphenols have been shown to modulate stem cell self-renewal, which has implications for disease management and prevention The present study was designed to investigate the primary effect of kahwah extract on murine mesenchymal stem cells and MCF-7 cell line by observing its effects on its viability, cytotoxicity, rate of cell proliferation, and migration along with its antioxidant potential observations of the present study demonstrated that Kahwah encumber the formation of mature adipocytes in the mesenchymal stem cells following exposure to adipogenic differentiation medium and inhibits the proliferation of MCF-7 cells upon administration of kahwah doses. Based on the observations, it is appropriate to claim that kahwah, having flavonoids majorly EGCG, possesses the ability to limit the proliferation of cancer cells and mesenchymal stem cells differentiation to adipogenic lineage. This could offer a logical strategy for creating kahwah as a flexible model for the treatment of obesity-related illnesses, such as carcinogenesis.

**Keywords**:Triple-negative breast cancer;adipogenesis;Kahwah;EGCG;green tea;MCF-7

## 1. Introduction

Obesity is a complex disease of great public health significance worldwide. It involves a few entanglements including diabetes mellitus type 2, cardiovascular dysfunction, and hypertension, and its predominance is

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expanding all over the world. Breast cancer is the driving cause of cancer-related mortality in women around the world. Concurring to the information from the Universal Organization for Investigate on Cancer, breast cancer alone accounts for 25% of all cancer cases and 15% of all cancer mortality among women, which is altogether higher compared with other cancers (Sung, Ferlay et al. 2021) The frequency of breast cancer in Asian nations have strongly expanded, with an anticipated ~2.5 million breast cancer cases in China by 2021(Ziegler, Anderson et al. 2008). A study conducted in the 1970s proposed that obese women were at a higher hazard of creating/developing breast cancer(de Waard and Baanders-van Halewijn 1974). Concurring to an orderly audit of epidemiological proof from the American Cancer Committee Inquires showed a large number of Animal models have illustrated that overweight status (obesity) significantly increases the risk of breast cancer(Wiseman 2008). Triple-negative breast cancer (TNBC), whiich accounts for 10-20 percent of all breast cancers has recently been defined as a special phenotype of breast cancer that does not overexpress the human epidermal growth factor 2 receptor (HER2) protein but does express the estrogen receptor (ER) and the progesterone receptor (PR). TNBC has a high rate of recurrence and distant metastasis, according to evidence from laboratory and observational studies, and poor overall survival (OS). Obese people produce a high level of tumor-promoting hormones such as leptin and estrogen, and a low level of the tumor suppressor hormone, adiponectin. Adipose tissues from tumor-bearing breasts, in particular, display a different molecular signature and physiological condition than those from tumor-free breasts. The association between obesity and breast cancer has not been fully known.

Our concentration should be towards normal food products that can re-establish each one of the capacities or help in keeping a solid way of life without debilitating the genuine secondary effects. The properties of spices can be investigated. It is apparent from history that spices are being used in food, beverages, and, surprisingly, drug ventures (Lai, Fu et al. 2012). They were additionally utilized in antiquated prescriptions to fix sicknesses (Kalalinia, Ghasim et al. 2018), as they are rich sources of polyphenols, flavonoids, terpenes, polysaccharides, lipids, and natural acids (Kakarala, Brenner et al. 2010)The polyphenols majorly modulate the proliferation and differentiation of normal stem cells (Akbarpoor, Karimabad et al. 2020)They have immunomodulatory impacts that explicitly inhibit or suppress the proliferation of cancer cells by stimulating the development of normal stem cells, empowering apoptosis, and initiating the outflow of defensive qualities that forestall the arrangement of malignant cells (Zhang, Lam et al. 2020)

Scientists have proactively shown the impact of different Spices, including their concentrates(extracts) as shown by (Huang, *et al.*2013) uncovered that cinnamic acid got from cinnamon (Cinnamomum zeylanicum), can impede proliferation and encourage apoptosis and further promotes differentiation, reduces the invasive ability of lung cancer stem cells.

Kahwah is perhaps the best up-and-comer as it contains a combination of different Spices so it could give more noteworthy helpful potential to human wellbeing. It is a rich source of antioxidants and different polyphenols(Farooq and Sehgal 2017)

Kahwah or Qehwah is traditionally used for treating headaches, digestive problems, and heart-related problems. Saffron, cardamom, cinnamon, clove, licorice, and green tea leaves the constituents used in the preparation of this drink exhibit various phytochemical properties. *Crocus sativus* (Saffron) is used as an antidepressant, respiratory drug, sedative, and carminative. Genoprotective, chemoprotective and antioxidant effects of the saffron extract were also observed in vitro (Jafari, Emami et al. 2022). *Elettaria cardomomum (*green or true cardamon*)*also shows antioxidant activity because of its constituents, kaempferol, quercetin, luteolin, and pelargonidin(Yahyazadeh, Ghasemzadeh Rahbardar et al. 2021). *Syzygium aromaticum* and *Camellia sinensis* also have well-documented benefits. *Cinnamon zeylanicum* one of the main components of Kahwah is being used since ancient times for its medicinal, antioxidant properties and anticancer activity(Guan, Su et al. 2016). Active compounds such as Camphene, phenol, epicatechin, and tannins attribute to the antioxidant activity of this spice. Cinnamomum cassia showed protection against $H_2O_2$- induced oxidative DNA damage in lymphocytes(Alsoodeeri, Alqabbani et al. 2020). The present study aims to explore the ability of Kahwah to demonstrate a potential source that can be used as an additional natural treatment for a variety of disorders. It also aims to determine whether or not high doses of various spices cause cytotoxicity and whether it shows effects on the phenotype of MCF-7 cells.

## 2. Materials and Methods

### 2.1. *Materials*

Murine Embryonic Mesenchymal Stem Cell Line C3H10T1/2 as well as Human Breast cell line MCF-7 were procured from NCCS Pune, Maharashtra, India and cells maintained in Dulbecco's Modified Eagle's Medium with heat inactivated serum and penicillin and streptomycin. Dulbecco's Modified Eagles Media (DMEM; #AL007A),

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Fetal bovine serum (FBS, #RM10832), Trypsin-EDTA solution (TCL007), Penicillin-Streptomycin (#A001), Dulbecco's phosphate buffer saline (#TS1006), Methanol, Ethanol, Dimethyl sulphoxide (# TC185), MTT reagent (#TC191), Trypan Blue (#TCL046), Hydrogen peroxide, all these chemicals were purchased from HiMedia Laboratories, Mumbai. All other laboratory reagents were obtained from local suppliers. Qehwah aka Kahwah has obtained from local buyer “the Tea heaven”.

### 2.2. Cell culture

Mesenchymal stem cells were maintained in Full growth medium (FGM) i.e. DMEM supplemented with 10%FBS and penicillin-streptomycin, at 37ºc in $CO_2$ incubator with 5%$CO_2$. All the experiments were further performed after 24 hrs so as to establish the cell monolayer marking it as 0-day. Experiments on MCF-7 cells were also performed post establishment of the cells monolayer in this same manner.

### 2.3. PREPARATION OF KAHWAH EXTRACT

The loose leaves were subjected to pulverization mechanically to obtain a fine powder. 5gm of this powder was taken and extracted for 2h at $80^0$C in 200ml of autoclaved distilled water using a magnetic stirrer. The solution was then filtered (Whatman paper no.1). The residue was then subjected to lyophilization. The lyophilized product was stored at $4^0$C to be used later. Stock solution was prepared of 1mg/ml concentration

### 2.4. DOSE AND TIME-DEPENDENT EFFECT OF KAHWAH ON C3H10T1/2

Morphological assessment of kahwah after administration of dosages were done after different intervals of time

### 2.5. TRYPAN BLUE EXCLUSION ASSAY

To check the viability of cells, 2ml of $1x10^5$ cells/ml per well were plated on 6 - well plate to create a confluent monolayer. The plate was Incubated for 24 hours at 37°C allowing the cells to attach and spread properly on the plate. After 24 hours of incubation, Kahwah extract was added from low to high dose and one well was kept as control as untreated cells. Cells were again incubated for 24 hours at 37°C in a humidified incubator. Number of viable cells were calculated using Haemocytometer. Trypan blue dye is intended to bind with the dead cells.Therefore, to determine whether cells absorb or exclude dye, 100µl of cell suspension from 1ml of cell suspension ( i.e. resuspended trypsinized cells in 1ml FGM. For preparing this the cells are trypsinized and then resuspended in 1ml FGM followed by centrifugation at 5000rpm for 5min at 4ºC. The supernatant is discarded and the pellet is resuspended in 1ml FGM) mixed with trypan blue dye solution (100µl), 10µl of this solution then subjected to the chamber, blue stained cells are counted under the microscope.

### 2.6. MTT ASSAY

The *in vitro* cytotoxicity is measured using an MTT assay. This assay measures the cell proliferation rate and conversely when metabolic events lead to apoptosis or necrosis, the reduction in cell viability is observed. $10^4$ Cells per 100µl Full growth media was added to the wells of the 96- well plate and incubated for the next 24 hours in tissue culture incubator at 37°C. Dosages of Kahwah extract of 60µl and 80µl
were added to the wells and the cell were incubated for 24 hours in a humidified incubator at
37°C. Then,100µl (0.5mg/ml) of MTT reagent was added and incubation was done for 4 hours at room temperature until apurple precipitate was visible. Subsequently DMSO (100µL) was added and readings were taken in an ELISA reader at an absorbance of 570nm.

### 2.7. LIPID PEROXIDATION ASSAY

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It is used to analyse the cellular oxidative stress Oxidative stress generated by $H_2O_2$ cell injury increases reactive oxidative species (ROS) levels in the cells that further degrade cellular lipids, majorly part of membrane systems, and leak out the cellular components in the lysate. During oxidation, ROS degrade lipids into unstable lipid peroxides that serve as indicators of cellular stress. 1 ml of $1x10^5$ cells/ml were plated in 24- well plate to create a confluent monolayer. Incubated plate for 24 hours at 37ºC allowing the cells to attach and spread on the plate. After 24 hours of incubation, $H_2O_2$ injury was given to cells adding of 100µM $H_2O_2$ for 30 minutes. Media was discarded after 30 minutes to stop the reaction and replaced with new FGM in each well after washing with DPBS. The Kahwah extract was added in doses 60µl and 80µl in the respective wells. One well was kept as control and marked as untreated cells. One well was kept as Hydrogen peroxide control ($H_2O_2$ control) without dose treatment. Cells were again incubated for 24 hours at 37ºC in humidified incubator. Next day, cells were then washed with DPBS, and 1ml trypsin was added to the cells. After 5-7 minutes incubation at 37ºC, 1ml of full growth media was added to stop trypsin reaction. Trypsinized cells were collected in Eppendorf tubes and sonicated 3 times for 5 second intervals at 40V setting over ice to prepare cell lysate. Then 2ml of TBA (thiobarbituric acid) was added to 1ml of this cell lysate in clean glass test tubes. Subsequently the these test tubes were then placed in boiling water for 60 minutes followed by immediate exposure in ice bath for 10 minutes. Centrifugation was then carried out for 4 min at 4º at 4000rpm and finally the readings were taken at 530nm by ELISA Reader. Dilutions of 500µM MDA were prepared for standard curve: 2µM, 4µM, 6µM, 8µM,10µM.

### 2.8. MESENCHYMAL STEM CELLS DIFFERENTIATION

Cells with density of 18000 cells/2ml per well in 6 well plate were incubated at 37ºC with 5% $CO_2$ for 48 h before initiating adipocyte differentiation. Then, the media was removed and cell monolayers were rinsed with room temperature DPBS. 2 mL (for 6-well plates) of adipocyte differentiation medium i.e. adipogenic cocktail (FGM containing 0.66µM Dexamethasone, 0.3mM IBMX (Isobutylmethylxanthine) and 0.66µg/ml insulin (Sottile et al, 2002) was added to each well to initiate adipocyte differentiation in the presence and absence of the 40µl, 60µl, 80µl doses (Kahwah extract). Later, on every 3rd day, the maintenance phase was initiated by carefully removing 2 ml of media from each well and replacing it with 2 ml of adipocyte differentiation medium with or without doses in each respective well for another 09 days until adipocytes reached full maturity (however on the 07th day, 2 ml of media used by cells was collected and preserved at 4ºC to be used later as Adipo conditioned media (Adipo CM) for further experiments with MCF-7 Cells). Subsequently the differentiation of Mesenchymal stem cells into adipocytes was assessed using the Oil Red O staining as described in the following section.

**Effect of Adipo conditioned media (Adipo CM) collected from dose treated and untreated C3H10T1/2 cells on MCF-7 cells**

100µl of MCF-7 cells with density of $1x10^4$ cells/100µl per well in full growth media were established in 96 well plate. After 36 hours, the pre-collected Adipo conditioned media (Adipo CM) from dose (Kahwah extract) treated and untreated **C3H10T1/2 cells** were administered (2µl per well) and incubated for 24 hours at 37ºC in humidified incubator and subsequently cell viability was determined using the aforementioned MTT assay.

### 2.9. OIL RED O STAINING

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To visualize the formation of lipid droplets and to asses the adipocytes maturation status The media was completely discarded from the wells with the help of an auto-pipette in a waste discard beaker. 1ml of 10% formalin was added into each well and incubated it at room temperature for one hour. Formalin was then removed from the wells using an auto-pipette and cells were washed with 1ml of 60% isopropanol per well. The wells were completely dried followed by the addition 1 ml of oil red O working solution ((1mg/1ml) per well and incubation at room temperature for one hour. The stain was removed and cells were then rinsed with distilled water. The wells were dried completely and finally the cells were viewed under phase contrast microscope at 10X and pictures were taken.

### 2.10. Triacylglycerols (TAG) QUANTIFICATION

The cells were fixed and stained using the steps as followed for Oil red O staining method as described before. The cells were then washed with water. The stained triglyceride droplets were extracted for 10 minutes with 1ml isopropanol and the absorbance was read at 510 nm spectrophotometrically .The triglyceride content was standardized by triolein.

### 2.11. TOTAL POLYPHENOLIC CONTENT

The total polyphenol content (TPC) was determined by using gallic acid as standard. The kahwa treated cell samples were mixed with 0.5 ml of 0.2 N Folin-ciocalteau reagents. After 5 min 2.0 ml of 20 % sodium carbonate was added. The absorbance of reaction was measured at 765 nm after 30 min of incubation at 37 ºC. The standard curve was prepared by solution of Gallic acid in methanol. The total phenolic content was measured in reference to gallic acid curve.

## 3. RESULTS

*Fig.A. yophilized kahwah extract*

*Fig.B.Solution of kahwah extract*

### 3.1. DOSE AND TIME-DEPENDENT EFFECT OF KAHWAH ON C3H10T1/2 cells

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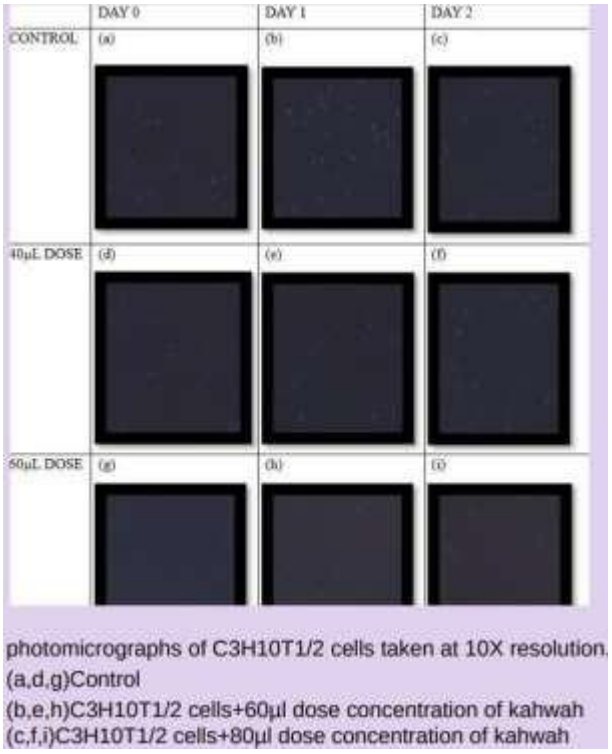


*Fig.C.*

Upon microscopic examination, on day 1 the control and the treated groups showed 80-90% confluency and the cells exhibited their characteristic morphology (Fig.C.). A similar observation was perceived on day 1 with no significant change in morphology or the cell number. However, on day 2 the cells exhibited a decrease in number in the control as well as the treated wells which may be due to the dislodging of the cells while handling the plates but comparatively the reduction in the cell number in the control well was less than that observed in the treated well. A slight change in the morphology of the cells was also observed on day 2. The results obtained in the present study are in line with the previous findings and well supported by the fact that Kahwah has an anti-proliferative effect as reported in some human cancer cell lines(Sudhakaran and Doseff 2020).

### *3.2 Viability and cytotoxicity assesment of* OF KAHWAH ON C3H10T1/2 cells *(mesenchymal stem cells)*

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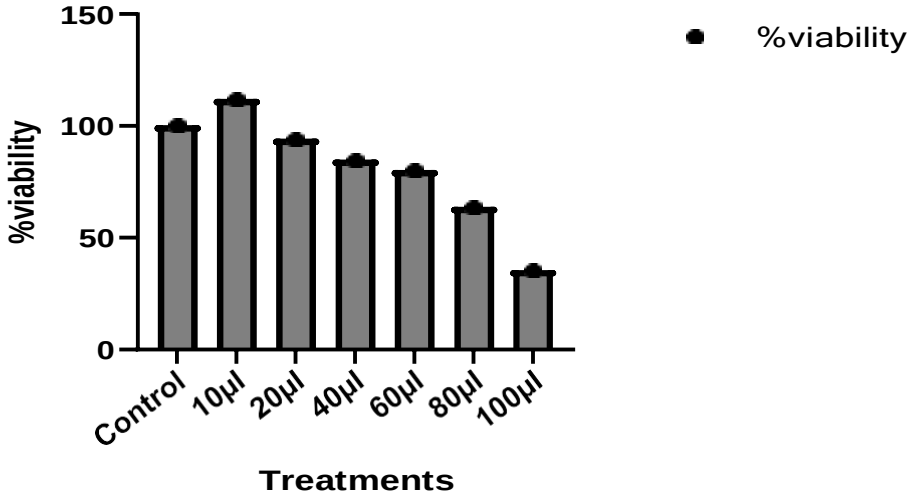


***Fig D:Graph representing the effect of kahwah administration at dose concentrations of 10µl,20µl,40µl,60µl,80µl, and 100µl 24 h post-treatment on percentage cell viability of C3H10T1/2 cells using trypan blue dye exclusion method***

The results obtained from the trypan dye exclusion test (Fig D) indicated that the cells which were administered with a 40µl and 60µl dose concentration of kahwah had a marginal decrease in the viability cells as compared to the untreated cells. These results indicate that at this dose concentration of KAHWAH administration, it did not exhibit a substantial decrease in the viability of the cells and would not confer cytotoxic effect on the cells. However, increasing the concentration of the extract may aggravate the cytotoxicity in the cells. Previous studies on kahwah suggest that a concentration of 100µM or higher would adversely affect the cell viability and may reduce it by 50% and more (Balasubramanian, Efimova *et al*. 2002). Measurement of cell viability and proliferation forms the basis for a wide range of *in vitro* assays of a cell population's response to external factors.

**The validation of the cytotoxicity of kahwah by trypan blue dye exclusion assay was checked by performing an MTT assay on C3H10T1/2 cells.**

In this assay, the amount of formazan crystals formed by the reduction of MTT dye by oxidoreductase enzymes in mitochondria, states how metabolically active the cell is, hence indirectly measuring the viability of cells.

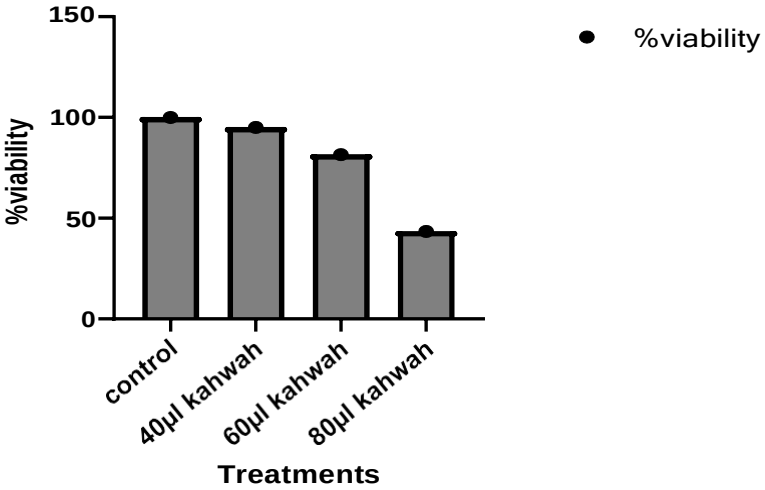

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**Fig E:*Graph representing the Cytotoxicity effect of kahwah administration at dose concentrations of 40µl,60µl,80µl 24h post-treatment using MTT assay on C3H10T1/2 cells***

As depicted in the Fig E, it was observed that cell viability was not significantly affected by the addition of kahwah and the rate of proliferation and cell viability was almost similar to the control for the lowest dose of kahwah as compared to the lower dose of kahwah i.e., of 40µl(95%) and 60µl(81.6%) respectively. However the higher dose of 80µl showed a (43.4%) cytotoxic effect.

Overall, it was observed that these doses of kahwah did not exhibit any significant cytotoxicity on C3H10T1/2cells. Further, to examine the effect of kahwah administration on the morphology and cell number of C3H10T1/2the surface morphology and cell number were studied through microscopic examination. The cells were plated in 6-well plates overnight and kahwah at doses concentrations of 60µl and 80µl were administered. Cells were allowed to grow at around 80-90% confluency and kahwah was administered whereas the control cells remain untreated throughout the experiment.

### *3.3. Kahwah possess antioxidant potential evident with the low level of oxidation in cells compared to the control*

Due to high phenolic content present in kahwah, it is even known for its anti-oxidant potential.Therefore, to analyze the anti-oxidant potential lipid peroxidation assay was performed. TBARS (Thiobarbituric acid reactive substance assaywas performed after 24 hours and readings were taken at OD-530nm, to check the extent of lipid peroxidation levels in the cells and compare the values of the treated and untreated groups. The results were as follows:

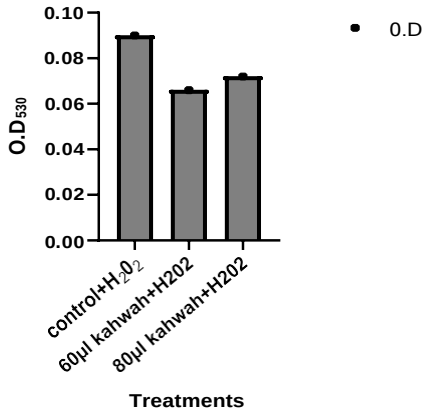


*Fig F:Graph representing the anti-oxidant effect of 60µl and 80µl dose concentrations of the kahwah as compared to the untreated groups on C3H10T1/2 using lipid per-oxidation assay*


*Fig G: Standard calibration curve of MDA*

The wells with the kahwah doses showed remarkably low levels of oxidation in the cells (Fig F). The wells with lower dose concentration of kahwah showed significantly low value as compared to that of the control and even the wells with high dose concentration of kahwah showed decreased oxidation levels as compared to the a control. The observations of the study are well supported with various reports, in the past which state that indeed kahwah does exhibit high antioxidant properties (Farooq and Sehgal 2017) which could be due to presence of EGCG in high concentration in kahwah and has been reported to have an anti-oxidant effect on oxidative stress in a mice model (Sung, Nah et al. 2000) which was linked with the capability of the polyphenols to modulate the antioxidant defense mechanism and decrease the lipid peroxidation in the cells.

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### *3.4. Formation of adipocytes was hindeed by kahwah doses, showing lesser amount of oil droplets assessing by oil red o staining in C3H10T1/2*

Adipogenesis was used as a paradigm in the present work to study effect of 40,60,80ul of the dose onto the MSCs along with the differentiation media. Exposure of these cells to the adipogenic differentiation media in the presence and absence of the doses was carried out. The MSCs change to adipocytes which have a specific morphology of spherical shape with accumulation of lipid droplets following differentiation.

**Fig H:Kahwah prevented lipid accumulation in differentiating C3H10T1/2 cells: The lipid accumulation in the cells undergoing adipogenic differentiation was analyzed by Oil red O staining following Kahwah treatment. Photomicrographs a), b) & c) represented control cells, cells treated with 60µl dose & cells treated with 80µl respectively.**

Such an inhibitory ability of kahwah on Adipogenesis was further validated by microscopic images of treated C3H10T1/2 cells captured after Oil red O staining in comparison to different dose treated cells (Fig H) and by measuring the triglyceride content through spectrometric analysis (510nm) of the extracted red stain color by isopropanol for 10min from the stained cells ( Fig I). The values are expressed as a percentage with respect to the control. Higher dosage of Kahwah shows lesser absorbance as compared to lower dosage as well as in comparison to the cells treated with adipogenic differentiation medium alone. These observations thus suggested that kahwah function as anti-adipogenic agent by preventing mesenchymal stem cell differentiation

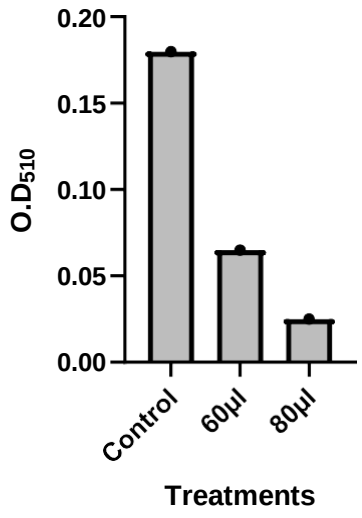


**Fig I:Graph depicting Inhibition of Triglyceride content in adipogenic differentiating C3H10T1/2 cells by Kahwah.. Since the red stain of Oil red O is indicative of the triglyceride content, its levels were observed by taking absorbance at 510 nm spectrophotometrically. The Kahwah treatment to the cells significantly reduced the absorbance of the extracted red color in comparison to the Cells exposed to differentiation medium only.**

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A linear calibration curve of gallic acid, with a coefficient of determination ($R^2$) value 0.989 was obtained. The total phenolic content is represented as gallic acid equivalents. The total phenolic content in the kahwa was measured to be 5.44mgGAE/g.

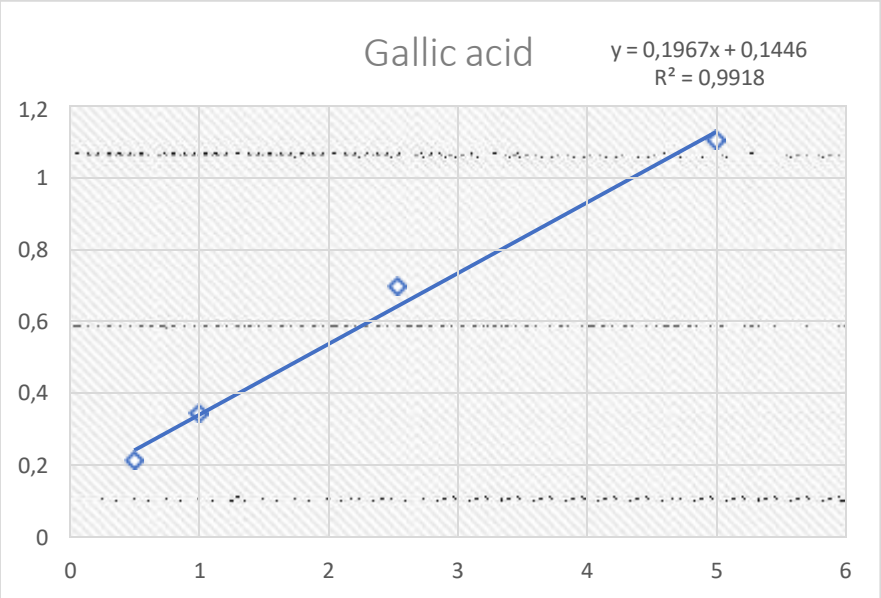


Fig J : standard gallic acid curve

### *3.5.* Adipo conditioned media (Adipo CM) collected from dose treated C3H10T1/2 cells *inhibits the proliferation of MCF-7 cells*

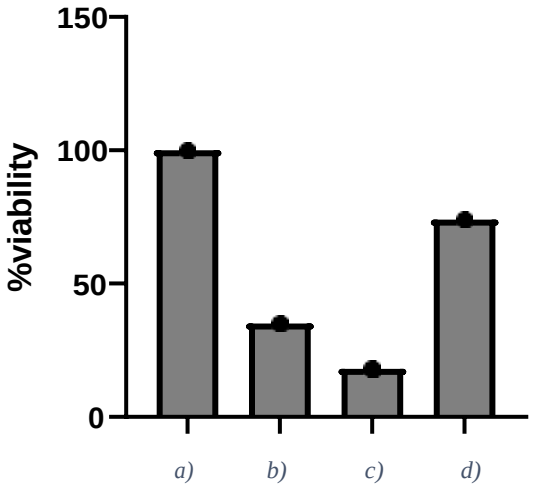


**Fig K:*Graph representing the cytotoxicity effect of a) control, b) Adipo CM collected from C3H10 T1/2 cells treated with 60µl kahwah plus adipogenic cocktail, c) Adipo CM collected from wells treated with 80µl kahwah plus adipogenic cocktail and d) Adipo CM without kahwah treatment on MCF-7 cell line***

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During adipogenesis, the conditioned media from ***C3H10 T1/2 cells treated with adipogenic cocktail with or without*** different doses of kahwah was collected after 7 days, so as to check whether Adipo CM triggers the growth of MCF-7 cells, and also to find whether adipo CM collected while **C3H10 T1/2 cells were treated with** kahwah doses triggers the growth of MCF-7 cells or inhibit it. In our study as shown in the Fig K, it was found that Adipo CM was able to increase the viability of MCF-7 cells but adipo CM containing kahwah significantly inhibits the growth of the MCF-7 cells. That shows that kahwah was able to alter the conditioned media profile of adipocytes. It gives the evidentthat obesity is somehow leading cause of developing cancer. As we have seen that Intervention of dietry polyphenols has proven to show beneficial effect on our health

**Discussion**

The present study was designed to investigate the effects of Kahwah on adipogenic differentiation of the mesenchymal stem cells. The observations of the present study demonstrated that Kahwah inhibited the lipid accumulation in the mesenchymal stem cells destined to differentiate in adipocytic cells following exposure to adipogenic differentiation medium. Besides its anti-adipogenic role, kahwah also prevented cell migration, an indirect marker for the cell proliferation(Punathil, Tollefsbol et al. 2008). Whether this anti-proliferative effect is akin to anti-adipogenic character of kahwah though warrants further study, but flurry of information lend support to this association. Based on the known pre-adipocyte murine cell culture models viz. 3T3-L1, 3T3-F442A and Ob17, it is known that upon reaching confluency and growth arrest, the opportunistic re-entry to cell cycle through hormonal induction led these pre-adipocytic cells to go through multiple cycles of post-confluence mitosis, called mitotic clonal expansion (MCE)(Klein and Ackerman 2003, Tang, Otto et al. 2003). It is rather a basic regimen of terminal adipocyte differentiation. As shown in the present study the inhibition of staining, restrained cell migration together with reduction in total cell number, by kahwah , advocates for its ability to prevent C3H10T1/2 mesenchymal stem cells differentiation into adipocytic lineage following hormonal cocktail exposure (Insulin, dexamethasone, 3- isobutyl- 1-methylxanthine). This cocktail is known to induce adipogenesis in various cell lines, including C3H10T1/2 cells(Pantoja, Huff et al. 2008). Earlier studies have shown that EGCG besides inhibiting the differentiation of pre-adipocytic cells also brings about wide range of biological functions like anti- oxidant, anti- cancer, anti-angiogenesis(Nathan 2003, Singh and Singh 2011). Previous reports describing growth arrest and differentiation of exponentially growing keratinocytes indicated that a dose of 50 µM and
100 µM of EGCG increased the conversion of undifferentiated keratinocytes into corneocytes with concomitant decreased cell proliferation(Balasubramanian, Efimova et al. 2002). This represents an important step in cells behavior to EGCG treatment. However, this certainly does not seem to be the universality, as in the present study concentration of 80 µL, 100 µL were found to be rather toxic to mesenchymal stem cells C3H10T1/2. Whereas, lower doses of kahwah 40 µL and 60 µL were sufficient reduce the proliferation of mesenchymal cells in a dose dependent manner. kahwah also reduced the migration of C3H10T1/2 cells as observed through scratch assay. Multitudinous reports(Hatzidaki, Papadimitriou et al. 2020) providing support to the fact that EGCG inhibits the proliferation and migratory behavior of proliferating cells further lend credibility to the observations of the present study. A growing body of work suggests that stem cells and cancer cell seems to possess some common molecular mechanisms (genomic/epigenomic/nongenomic) for retaining their characteristics off course deciding controlled or deregulated proliferation(Taipale and Beachy 2001).The inhibition of proliferation (migration in scratch area) of stem cells by kahwah may plausibly target similar molecular events as observed in cancer cells. These observations are also corroborated by the fact that kahwah treatment reduced the cell count significantly further suggesting that kahwah might be regulating cell proliferation which is an important character for long

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term maintenance of cell pleuripotency whereby besides anti-proliferative action, it also reduces adipogenesis. The anti-obesity role of kahwah further gets corroboration from past studies(Klaus, Pültz et al. 2005, Murugan 2007) though the precise mechanism remains elusive. Present results showed that kahwah inhibited the differentiation of C3H10T1/2 cell lines and so it can be used as supplement for controlling the obesity which is responsible for associated diseases like diabetes and heart problems. Thus, it is possible that as in pre-adipocytic cells, the kahwah treatment to mesenchymal stem cells also follows such a paradigm to inhibit the Adipogenesis.

Based on these observations, it is appropriate to claim that kahwah, having flavonoids majorly EGCG, possesses the ability to limit the mesenchymal stem cells differentiation to adipogenic lineage. This may provide a rational approach towards developing kahwah as a versatile molecule for the intervention of obesity related disorders, including carcinogenesis.

More studies will be needed to identify, among the various spices, which one(s) have a leading role in order to design pharmacological interventions.

**Conclusion**

In conclusion, our study first validated modulation of cytotoxicity and viability by kahwah doses as confirmed by trypan blue exclusion assay followed by MTT assay. Kahwah didn't show any notable morphological disruption despite showing slight toxicity at higher concentration. Formation of mature adipocyte was deter by different doses of kahwah, high dose of kahwah did not show any slight differentiation of mesenchymal stem cells in to mature adipocytes. Validated by staining (oil red o stain) and TAG quantification. Adipo conditioned media (Adipo CM) collected from dose treated C3H10T1/2 cells inhibits the proliferation of MCF-7 cells. This final piece of data strongly shows that, although it is beyond the immediate purview of this work, mature adipocytes conditioned media may provided the favorable environment which triggers the proliferation of MCF-7 cells while kahwah was able to modulate that environment hence inhibit the growth of MCF-7 cells. Although the current methodology, which uses the cells' conditioned media to reflect the synergistic action of conditioned media, better reflects the pathophysiological tissue microenvironment, the precise identification of factors which lead to these modulations would be a logical next step. Since hypoxia and EGCG tissue biodistribution/bioavailability continue to be key concerns in obesity, one may consider addressing them in the future.

**Declaration of Competing Interest**

None

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