THE STUDY OF ACUTE AND CHRONIC EXPOSURE OF ARSENIC ON TRANSFORMATION OF HaCaT CELLS

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MASTER OF PHILOSOPHY

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled as **"The study of acute and chronic exposure of arsenic on transformation of HaCaT cells"** submitted for the award of **Degree of Master of Philosophy** has been carried out by **Mr. Mohit Rajput** under the guidance and supervision of Prof. Rana Pratap Singh at the School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India.

This work is original and has not been submitted so far, in part or in full for award of any other degree or diploma of any other university.

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Dedicated to my lovely family...

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Abbreviations

AAR	Age adjusted rate
ABC	ATP binding cassettes protein
APL	Acute promyelocytic leukemia
AQPs	Aquaglyceroporins
As	Arsenic
As^{III}	Arsenite
As^{V}	Arsenate
AS3MT	Arsenic (+3 oxidation state) methyltransferase
ATO/As_2O_3	Arsenic trioxide
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BCC	Basal cell carcinoma
BER	Base excision repair
CDK	Cyclin-dependent kinase
CML	Chronic myelogenous leukemia
COX	Cyclooxygenase
DCFHDA	Dichloro-dihydro-fluorescein diacetate
DDR	DNA damage response
DEPC	Diethyl pyrocarbonate
DMA^{III}	Dimethylarsinous acid
DMA^{V}	Dimethylarsinic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
EDTA	Ethylene-diamine tetra acetic acid
EGFR	Epidermal growth factor receptor

EPA	Environmental Protection Agency
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
FDA	Food and Drug Administration
Fs	Fisetin
GSH	Glutathione
GST	Glutathione s-transferase
Gy	Gray
H_2O_2	Hydrogen peroxide
HaCaT	Human adult, calcium, temperature
IARC	International Agency for Research on Cancer
JNKs	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MDR	Multi drug resistance
MMA^{III}	Monomethylarsonous acid
MMA^{V}	Monomethylarsonic acid
MMP	Matrix metalloproteinase
MRP	Multidrug resistance protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide
NCCS	National Centre for Cell Science
NCRP	National Cancer Registry Programme
NMSC	Non-melanoma skin cancer
NSAIDS	Non-steroidal anti-inflammatory drugs
NSCLC	Non-small cell lung cancer
ODC	Ornithine decarboxylase
ODD	Oxidative DNA damage
PBS	Phosphate buffered saline

PCA	Prostate cancer
ppb	Parts per billion
ppm	Parts per million
ROS	Reactive oxygen species
SAM	S-adenosylmethionine
SCC	Squamous cell carcinoma
SFM	Serum-free media
TGF	Transforming growth factor
UVR	Ultraviolet radiation
WHO	World Health Organization

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Introduction and Review of Literature

1. Introduction

Cancer is a multifactorial disease which is characterized by uncontrolled cell division. It has no specific origin since it can arise almost anywhere in the body. In normal tissue architecture, cells keep on dividing to replace the old and dead cells in order to maintain the integrity and functionality of the organ. Sometimes, this orderly sequence of events of cell division gets disrupted, that leads to uncontrolled cell division and finally tumor formation. Tumor is a mass of continuously dividing cells. It becomes malignant in nature when it starts metastasizing to other parts of the body through blood or lymph system to form a secondary tumor at new site. The genetic material of cancer cell harbors mutations that render them to behave different from the normal cells. There are many types of cancers depending upon the origin of tissue types such as carcinoma, sarcoma, leukemia, multiple myeloma and melanoma. Carcinoma is the most common type of cancer of epithelial cells. Cancer is one of the leading causes of deaths worldwide. The understanding of knowledge of its cause is important for its early diagnosis and better therapeutics. The changes in lifestyle and environment in last few decades are one of the main factors for increasing incidence of cancer all over the world. However, according to published report, the most common cause of cancer related mortality is due to tobacco consumption (25-30%), diet (30-35%) and infection (15-20%) and remaining percentage of mortality is due to radiation, stress, pollutants etc (Anand et al., 2008).

According to the report published in Global Cancer Facts and Figures, 2015, it has been estimated that about 8.2 million cancer deaths and 32.6 million peoples have suffered with cancer worldwide in 2012, with 14.1 million new cancer cases (Ferlay et al., 2015). According to Global Cancer Facts and Figures, (2015), 21.7 million new cancer cases and 13 million deaths have been predicted till 2030. In developed countries, prostate cancer is the most common one in men, however, lung cancer is the most common type of cancer occurring globally.

According to GLOBOCON report, 2012, in India, lung, lip and oral cancers are the most common cancer occurs in male while in female, breast cancer is the most common, alongwith differences in mortality rate with cancer in both sexes. According to the report published by National Cancer Registry Programme (NCRP), in India, the Aizawl district of Mizoram shows the highest Age Adjusted Rate (AAR) in both male and female. In males, the cancers of lung, mouth, oesophagus and stomach are common while in females, cervix and breast cancer are the common one in all reported cases.

According to World Health Organization (WHO) report, the global incidence of non-melanoma skin cancer (NMSC) occurs between 2 to 3 million every year while 132,000 cases of melanoma skin cancer occur globally. However, most of the skin cancer related deaths have been due to melanoma. It is estimated that new cases in males and females would be 42,670 and 31,200 respectively, in 2015 and estimated deaths were 9,940 (Cancer facts and figures 2015). In India, the statistics for skin cancer is not available. But the occurrence of NMSC in Asians is rare, however, various studies have reported that the squamous cell carcinoma (SCC) is the most common skin cancer that occurs in India (Panda, 2010).

The most common cause of skin cancer is ultraviolent radiation (UV) from sun exposure (Saladi and Persaud, 2005). There are also many other factors that increase the person's risk for getting skin cancer such as family history, medical history, use of tobacco and exposure to chemicals such as arsenic and coal tar (American Academy of Dermatology). There are many reports which have established the relationship between arsenic and skin cancer over a past few decades. Chronic ingestion of arsenic through drinking water over a long period of time in a dose-response relationship induces skin cancer. Arsenic has been known to cause three common malignancies in patients i.e. Bowen's disease, squamous cell carcinoma and basal cell carcinoma. The appearance of malignancies caused by arsenic exposure varies from 10 years in Bowen's diseases to 20 to 30 years latency period in other types of skin cancers (Martinez et al., 2011). Arsenic tends to accumulate in skin which leads to condition known as hyperkeratosis, a hallmark of the chronic exposure of arsenic. There are many clinical manifestations of chronic arsenic poisoning, most commonly observed symptoms include arsenical skin lesions, melanosis, conjunctivitis and keratosis (Basu et al., 2001). In India, especially in West Bengal, millions of people are facing this problem due to their dependency of arsenic contaminated groundwater for drinking and other household purposes. The information on molecular mechanism of transformation of normal cell into malignant caused by arsenic exposure is very limited which needs to be explored.

Chemoprevention is the use of natural or synthetic compound which suppress the cancer malignancy. Many chemoprevention based studies have been done in different types of cancer such as breast, prostate and colon cancer (Steward et al., 2013). Skin carcinogenesis is a multi-event process which is affected by many factors. The chemoprevention studies on skin cancer are known although more studies need to be done in this area. Many

phytochemicals are known to play important role in skin chemoprevention such as silibinin, resveratrol, curcumin and anthocyanin (Singh et al., 2014).

The incidence of skin cancer is increasing every year. The role of arsenic in skin carcinogenesis is very prominent and well-studied. The exposure of human to arsenic is mainly though water and food, since most of the population around the globe depends on groundwater for drinking purpose. But the mechanism of arsenic induced skin carcinogenesis is not well studied at molecular level. Arsenic affects the HaCaT cells, which represents skin keratinocytes, at both low as well as high concentration. In our studies, we have assessed the effect of arsenic at lower concentrations which are non-cytotoxic, and induce the proliferation of HaCaT cells. Further, the effects of phytochemicals, silibinin and fisetin, on arsenic-transformed cells HaCaT cells were examined to mitigate the effects of arsenic. We also explored the molecular alterations caused by arsenic in HaCaT cells involved in transformation of cells, which might cause malignant transformation of cells.

Overall, we have found that the low concentrations of arsenic transformed the HaCaT cells which were associated with several molecular alterations. We have also examined the efficacy of phytochemicals, silibinin and fisetin, against transformed HaCaT cells and observed that they reduced the effect of arsenic-induced transformation. So, further studies are needed to be done at lower concentrations of arsenic so that its effect could be further explored at molecular level. Therefore, deciphering of mechanisms of arsenic-induced transformation could provide insight into arsenic- mediated skin carcinogenesis in general population exposed to arsenic continuously through drinking water. These molecular alterations associated with arsenic transformation of HaCaT cells could provide novel targets for the development of novel therapeutics against skin cancer.

2. Review of literature

The study of cancer is known as oncology. Development of cancer is a multistep process in which abnormal cells continue to divide without regulation. Cancer cells migrate to other parts of the body and grow there but its origin remains the same. For example, if breast cancer cells migrate to liver and colonize there, it is still considered as breast cancer. Cancer could be solid and liquid but majority of the cancers are solid in nature. Cancer of blood forming cells (leukemia) does not form the solid tumor. Also, not all the tumors are cancerous in nature (Sudhakar, 2009).

Cancer has a long history. The Greek physicians Hippocrates (460–370 B.C) used cancer word, which was taken from Greek words *Karkinos*, to describe the carcinoma tumors. But he was not the first one to discover cancer. Around 1600 BC, there are some bodies of mummies in Egypt found to have bone cancer. There are several theories given over a period of time to explain cancer. The Humoral theory was given by Hippocrates which was prevalent in middle age for 1300 years. Later on some other theories like lymph theory, blastema theory, chronic irritation theory and trauma theory were given for cancer. In 18^{th} century, parasite theory was given, where scientists believed that cancer is contagious and parasites are responsible for its transmission. Later on, in 1911 Peyton Rous discovered the type of cancer in chickens that is caused by a rous sarcoma virus. During middle of 20^{th} century, with the discovery of deoxyribonucleic acid (DNA) structure, scientists explored more complex biology behind the cancer. In 1970, further studies lead to the discovery of critical family of genes involved in cancer i.e. tumor suppressor gene and oncogene. With the advancement of technology, scientists explored deeper aspects of cancer and with the growth of knowledge of various cancer, now it is possible to screen cancer at early stage and prevent it in most cases (Sudhakar, 2009).

2.1. Worldwide statistics of cancer

There are many organizations which are maintaining the statistical records of occurrence of cancer. These statistics help in finding the incidence, mortality rate and geographical distribution of cancer all over the world. According to the report published in Global Cancer Facts and Figures, 2012, the cancer causes one death out of seven deaths worldwide. Cancer causes more deaths than AIDS, tuberculosis, and malaria combined. It has also been reported that in high income countries, cancer is the second leading cause of death and in low and middle countries, cancer is the third leading cause of deaths following cardiovascular diseases and infectious and parasitic diseases. It has been estimated that

about 8.2 million cancer deaths and 32.6 million peoples have suffered with cancer worldwide in 2012. Also, in 2012, 14.1 million new cancer cases have been reported (Ferlay et al., 2015).

According to Global Cancer Facts and Figures, 3rd edition (2015) which is produced by American Cancer Society (ACS) in collaboration with International Agency for Research on Cancer (IARC), 21.7 million new cancer cases and 13 million deaths have been predicted till 2030. Lung cancer is the most common type of cancer occurring globally, however in developed countries, prostate cancer is the most common one in men. Among women, breast cancer is the most common cancer occurring in developed as well as developing countries. On the contradictory, lung cancer is the leading cause of death in women in developed countries. Infections are one of the reasons for the cancers across the globe. The statistics for cancers caused due to infections vary for developed and developing nations i.e. 7% and 23%, respectively. The two main types of cancer due to infections in man and women are liver and cervix, respectively, with stomach cancer common in both the sexes in developing nations.

It is reported recently that the cancer incidence rates are declining in rich countries as contrast to poor countries where incidence are rising (Torre et al., 2015). The incidence of cancer and estimated deaths all over the world in male and female have been represented in pie chart form as shown in **Fig.1,** which is adopted from GLOBOCON, 2012.

2.2. Statistics of cancer in India

The mortality rate due to cancer is the second most common after cardiovascular diseases all over the world. Cancer deaths account for 23% and 7% in USA and India, respectively. Also, the incidence of cancer is increasing over a period of time in developing countries (Ali et al., 2011). According to GLOBOCON report, 2012, in India, the most common cancer occurs in male is lung, lip and oral cancers while in female, breast cancer is the most common one. As well as the mortality rate with cancer in both sexes is different. Cancer mortality rate in male is more because of lung cancer while in female breast cancer causes more mortality. The various statistics of cancer in India is shown in **Fig.2**.

According to the report published by National Cancer Registry Programme (NCRP), Indian Council of Medical Research (2006-08) (ICMR), in India, the Aizawl district of Mizoram shows the highest Age Adjusted Rate (AAR) in both male and female. In males, the cancers of lung, mouth, oesophagus and stomach are common in registries while in females cervix and breast cancer are common in all reported cases. The incidence of cancer in India is

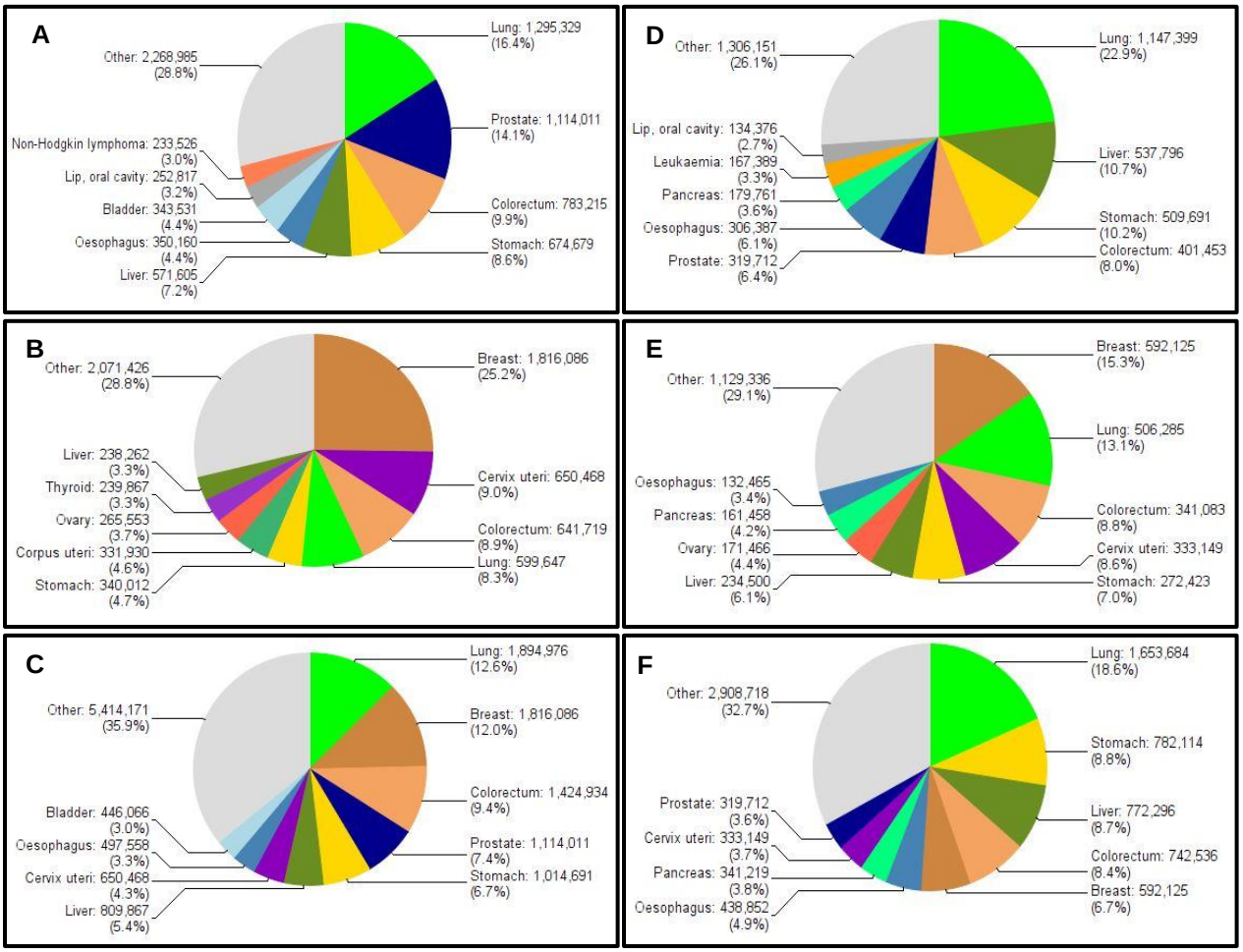


Figure 1. Estimated age-standardised incidence and mortality rates of various cancers worldwide. (A), (B), (C) Showing estimated age-standardised incidence rates for men, women and both sexes, respectively. (D), (E), (F) Showing estimated age-standardised mortality rates for men, women and both sexes, respectively. SOURCE: GLOBOCON 2012 (IARC)

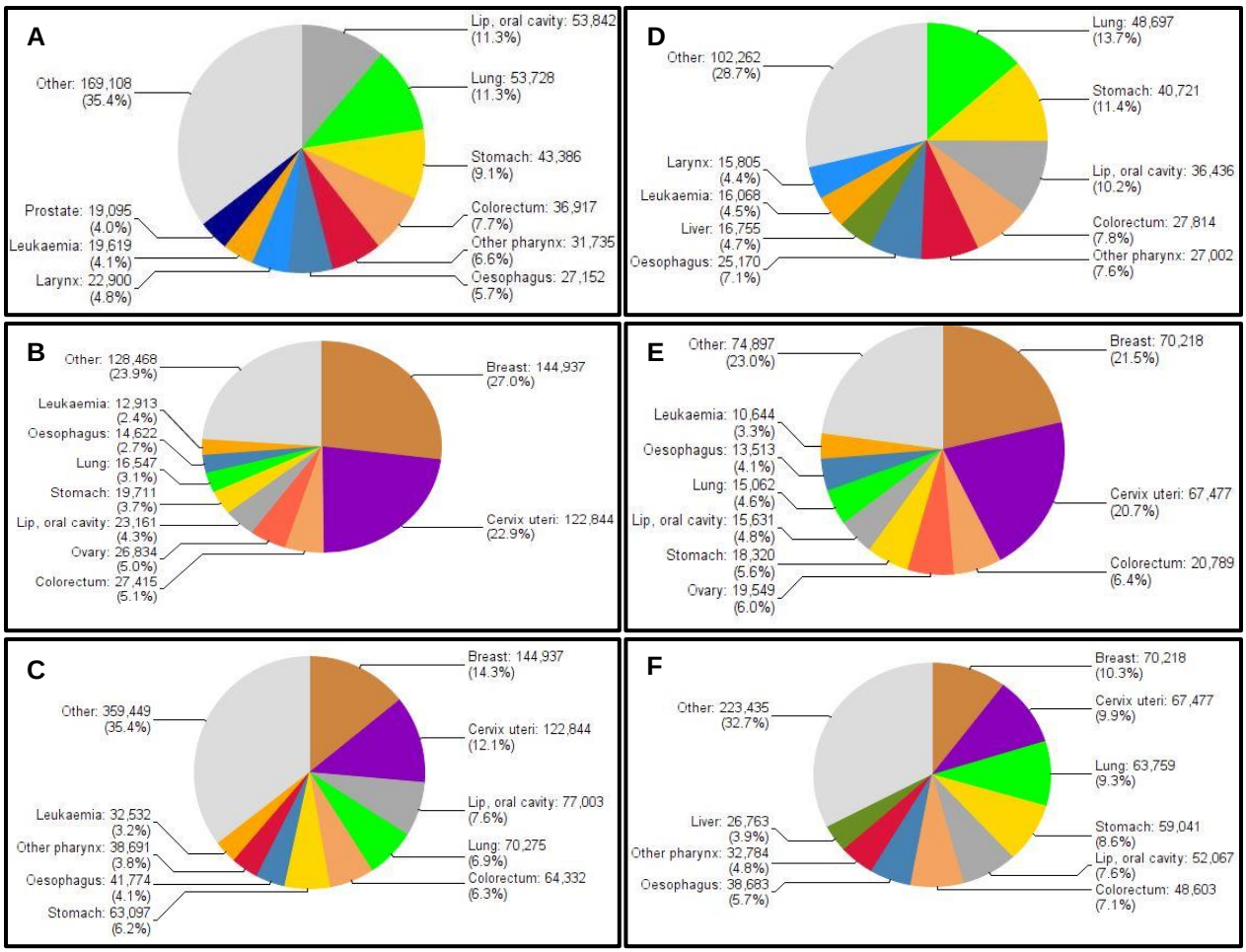


Figure 2. Estimated age-standardised incidence and mortality rates of various cancers in India. (A), (B), (C) Showing estimated age-standardised incidence rates for men, women and both sexes, respectively. (D), (E), (F) Showing estimated age-standardised mortality rates for men, women and both sexes, respectively. SOURCE: GLOBOCON 2012 (IARC)

found to be lower when compared to other western countries but in future the incidence is expected to rise because of changes in lifestyle and migration of population from rural areas to the urban areas. Dietary factors and lifestyles also play role in increasing incidence of cancer.

2.3. Causes of cancer

Since, cancer is a complex disease, there are various factors responsible for its occurrence. The changes in lifestyle and environment in last few decades are one of the main factors for increasing incidence of cancer all over the world. The cause of cancer could be genetic and non-genetic. However, the genetic cause of cancer accounts only for 5-10% of all cancers and 90-95% cancers are due to changes in environment and lifestyle. The changes in lifestyle include diet, cigarette smoking, alcohol, sun burn, pollutants, infections, stress and obesity. According to published report, the most common cause of cancer related mortality is tobacco consumption (25-30%), diet (30-35%) and infection (15-20%) and remaining percentage of mortality is due to radiation, stress, pollutants etc (Anand et al., 2008).

2.4. Statistics of skin cancer in India and worldwide

Skin is the largest organ system of the human body. The basic structure of skin includes outer epidermis layer, middle dermis layer and the innermost hypodermis. The dermis layer of skin has nerves, blood vessels, sweat gland, oil glands and contains cells such as mast cells and fibroblast cells. The epidermis layer consist of three different types of cells i.e. squamous cells, basal cells and melanocytes, each one has their own characteristics and specialized function. Cancer of squamous and basal cells, is known as non-melanoma skin cancers (NMSC) and in melanocytes it is known as melanoma.

According to World Health Organization (WHO) report, the global incidence of NMSC occurs between 2 to 3 million every year while 132,000 cases of melanoma skin cancer occur globally. The incidence of both skin cancers increasing every year and thus posing a threat in near future. Skin cancer is one of the most common cancer occurring worldwide mainly in fair skin individuals. The worldwide incidence of skin cancers is not reported anywhere because it is highly curable in nature. According to the report in US, there were 3.5 million cases of Non-melanoma skin cancer (NMSC) diagnosed, and 2.6 million cured in 2006. However, most of the skin cancer related deaths have been due to melanoma. It is estimated that new cases in males and females would be 42,670 and 31,200 respectively, in

2015 and estimated deaths were 9,940 (Cancer facts and figures 2015). In India, the statistics for skin cancer is not available. But the occurrence of NMSC in Asians is rare. Also, from various studies, it has been reported that the squamous cell carcinoma (SCC) is the most common skin cancer that occurs in India (Panda, 2010).

2.5. Skin cancer and its causes

Skin cancer is one of the most common cancers occurring worldwide. It is less aggressive cancer in which skin cells which normally divides undergoes transformation and thus divides without regulation. Although the incidence of skin cancer is high, but the threat of serious consequences due to skin cancer is less. Unlike other cancers, the metastasis property of skin cancer cells is limited. Skin cancer has been categorized into different types depending upon the type of skin cells in which cancer has been occurred. Cancer of skin cells of epidermis i.e. basal and squamous cells, collectively known as Non-Melanoma Skin Cancer (NMSC) and the cancer of melanocytes is known as Melanoma. Basal cell carcinoma (BCC) is the most common cancer and squamous cell carcinoma is the second one. But melanoma is fatal, although it is less common than other types of skin cancer.

The most common factor that lead to damage of skin cells is to get exposed from UV rays from sunlight. Ultimately, this damage of skin cells promote and make them prone to skin carcinogenesis. The long-term or short-term exposure from sunlight also affects the consequences of UV light on skin cells. The UV rays coming from sunlight consists of UVA, UVB and UVC. But, the UVB ray is the major cause for skin cancer. There are many risk factors which increase the chances of an individual getting skin cancer. These includes fair skin, family history (genetic), intimacy of sunburn, geographical distribution, moles, weakened immune system, exposure to radiation and chemicals. The chemical pollutants include heavy metals such as arsenic, benzene, benzopyrene and ozone (Baudouin et al., 2002). There are certain skin related medical complications most likely to increase the chances of getting skin cancer such as psoriasis, actinic keratosis, xeroderma pigmentosum, gorlin's syndrome and eczema.

2.6. Arsenic

Arsenic is a natural metalloid element that is commonly found in our earth's crust in various forms. It is ubiquitously found in our environment. Arsenic occurs in both free as well as mineral form. It is usually found in combination form with other metals such as sulphur or oxygen. There are many arsenic ores present in nature but common ones are arsenopyrite

(grey arsenic), realgar or sandarach (red arsenic), orpiment (yellow arsenic) and arsenolite (white arsenic).

2.6.1. History of arsenic

Arsenic has a long history as it was used as a secret weapon for homicidal activity in early period to kill emperors. It is believed that the arsenic was first identified by Albertus Margus, a German scholar around 1250 AD. Arsenic is poisonous is nature, and it is well known as "the king of poisons". The purpose of its use as a poison is mainly lie in its properties such as lack of odor, color or taste when mixed in edible things. Also, symptoms caused by arsenic more or less similar to food poisoning and difficult to detect.

2.6.2. Uses of arsenic

Arsenic is well known element since ancient time and used as 'the healer' as well as 'the poisoner' in early period. Arsenic also has medicinal history. Hippocrates used arsenic to treat ulcers and abscesses. Even, traditional Indian and Chinese medicines used arsenic for treating diseases. Paracelsus was the first who gave precise directions for the use of arsenic as a therapeutic agent. One percent (1%) potassium arsenite solution also known as fowler's solution, developed by Thomas Fowler, was found to be effective in treating malaria and trypanosomiasis. The fowler's solution was also used for treatment of various ailments such as asthma, eczema, anemia, hypertension, gastric ulcers and tuberculosis. Arsenic was also used to treat many cancers. In 1878, this solution was used to treat chronic myelogenous leukemia (CML) where it was found to reduce the white cell count in individuals (Frith, 2013). In the early of 20^{th} century, more therapeutic ability of arsenic came to limelight when *Paul Ehlrich* developed an organic arsenical known as salvarsan for treating syphilis and trypanosomiasis. In 1930, the use of arsenic for treating CML became popular because of its effectiveness against leukemia (Antman, 2001). Nowadays, for treatment of relapse cases of acute promyelocytic leukemia (APL), subtype of acute myeloid leukemia (AML), FDA has approved the use of arsenic trioxide, As_2O_3 (ATO) (Tallman et al., 2015). Arsenic based pesticides such as lead arsenate and copper arsenate is widely used in agriculture in a controlled way for effectively controlling the damage caused by insects.

2.6.3. Geographical distribution of arsenic in worldwide

Arsenic is a naturally occurring element which is found ubiquitously in nature. Arsenic could be found in both inorganic as well as in organic form. Inorganic form of arsenic is usually found in soils, sediments and groundwater while organic form of arsenic mainly occur in seafoods such fish and shellfish. There are many arsenic species existing naturally i.e. arsenate, arsenite, monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V). There are various oxidation states of arsenic such as arsenic (0), arsenite (As^{III}), arsenate (As^V) and arsine (-III) existing in the nature. The occurrence of different forms of arsenic varies. Arsenate is the most prevalent form present in water as compared to arsenite. Also, in aerated water, arsenite is oxidized to arsenate form at high pH. The organic form of arsenic such as MMA and DMA occurs in very small amount in surrounding. Most of the observed pathological conditions due to arsenic has been associated with inorganic form of arsenic (Centeno et al., 2002).

The factor affecting the distribution of arsenic at any region depends on the sources and geological condition of that region. The sources of arsenic in any region could be anthropogenic as well as natural. The use of arsenical pesticides in agriculture, industrial waste etc is the common source of arsenic and geological conditions of the area is natural contributing factor. The groundwater contamination with arsenic affects many south asian countries such as India, Bangladesh, Pakistan, Nepal, Myanmar and China. These further effects the population residing there because they mainly depend on the groundwater for household uses. But the arsenic level in groundwater in Pakistan was found to be lower when compared to India and Bangladesh. The range of arsenic in groundwater varies from one place to another. The highest range of arsenic in drinking water in Pakistan found to be 350 µg/L while in other countries such as, Bangladesh and Nepal, the amount of arsenic is significantly higher (1700-2500 µg/L) (Fatmi et al., 2009)**.** There are several other countries which are also facing the crisis of arsenic in drinking water namely, USA, Mexico, Taiwan, Chile, Argentina, Poland, Canada, Hungary and Japan. The occurrence of arsenic in groundwater in West Bengal, India is considered as biggest calamities in the world (Jain and Ali, 2000)

A large population all around the world is facing arsenic crisis because of their dependency on the contaminated groundwater. This worldwide problem mostly impacts the population of poorest regions in the world, affecting around 226 million people. But, among all arsenic affected regions, the most critical areas are Argentina, Chile and Mexico (Bardach et al., 2015). The natural presence of arsenic in sediments and soils is because of the affinity of

arsenic to solid matters alongside river. Also, arsenic conjugated with sediments do not harm the nature as they are not present in water. But, certain environmental pollutant changes the condition, such as change to alkaline pH, which promote arsenic release from the sediments and thus leads to contamination of water (Gutierrez et al., 2009). The **table 1** enlists selected severely affected regions with arsenic in groundwater amount and permissible range given by WHO.

2.6.4. Indian scenario with respect to arsenic

Arsenic has become a global concern from past few decades, which is affecting the large number of the population of the world as shown in **Fig.3**. In India, the concern for arsenic contamination over a period of time has increased with many research studies reporting the presence of high amount of arensic in groundwater. The first report was published by Garai *et al* in 1983, which reported the incidence of arsenic contamination in groundwater in many villages of West Bengal.

Country	Region	Ground level As level (in ppb)	Permissible Limit (in ppb)
Bangladesh	**Noakhali**	**<1-4730**	**50**
China	**-**	**50–4440**	**50**
Greece	**Fairbanks (mine tailings)**	**Up to 10,000**	**10**
India	**West Bengal Uttar Pradesh**	**10–3200**	**50**
Nepal	**Rupandehi**	**Up to 2620**	**50**
Thailand	**Ron Phibun**	**1–>5000**	**10**
USA	**Tulare Lake Red River Delta**	**Up to 2600**	**10**
Vietnam	**Mekong Delta**	**<1–3050**	**10**

Table 1. List of countries with level of arsenic found in groundwater (Adopted from (Shankar et al., 2014).

Figure 3. Map depicting the geographical distribution of arsenic worldwide. Arsenic enters in groundwater through natural or anthropogenic sources. Arsenic affected aquifers found worldwide. Mining is one the important anthropogenic factors that contributes entry of aresnic in environment. (Adopted from Smedley, P. L. (2008)."Sources and distribution of arsenic in groundwater and aquifers").

These studies also showed that many districts in Bihar like Bhojpur, Patna, Kishanganj and Bhagalpur have arsenic content in groundwater above the permissible limits i.e. greater than 50 µg/ml **(Kumar A et al.,)**. In India, the river basin of the Ganga, Brahmaputra and Meghna is the most affected region and a significant population is affected by arensic. Although, most of the studies regarding arsenic contamination in groundwater have been done in various parts of the West Bengal, there are many others states facing these natural calamities such as Assam, Chhattisgarh, Bihar, Uttar Pradesh, Jharkhand and Andhra Pradesh (Guha Mazumder and Dasgupta, 2011).

The use of arsenicals pesticides in agriculture is one of the major anthropogenic factor for increasing arsenic content in groundwater. In West Bengal, many areas uses the arsenic contaminated water for irrigation of rice plants, but studies have shown that the amount of arsenic in rice grain has not exceeded the permissible range given by WHO (Bhattacharya et al., 2010).

2.6.5. Standard recommendation of arsenic in drinking water given by WHO

The adverse effects of arsenic on human health have been observed in large number of population across the globe, mainly in the population which depends on arsenic contaminated groundwater for their household uses. The studies have shown that the effect of long term consumption of arsenic at higher amount is very fatal. The epidemiological studies have shown that out of 10 people one will die of cancer if has been exposed through drinking water contained arsenic greater than 500 µg/L over a period of one year (Smith and Smith, 2004). Initially, when the little facts were known about the arsenic related toxicity, the US public health services in 1942 set the maximum limit of 50 µg/L of arsenic in drinking water. Till 1984, the other US agency Environmental Protection agency (EPA) and WHO follow the same standards of 50 µg/L as maximum permissible limit. After recommendations of these guidelines, still there were reports of arsenic related toxicity that led to the revision of standards and 10 µg/L (10 ppb) was set as maximum permissible limit recommended by WHO (Ning, 2002). The national level standards for arsenic limit in drinking water vary within a country as shown in **table 2.** The WHO recommendation has been adopted as a standard by many nations. But many countries like India, Bangladesh and China still follow the earlier guideline recommended by WHO of 50 µg/L as a maximum permissible limit for arsenic (Yamamura 2001).

Arsenic Standard	Countries Adopted
Countries following standard < 10 µg/L	**Australia**
Countries following standard 10 µg/L	**European Union, Japan, Jordan, Syria**
Countries following standard < 50 µg/L	**United States, Mexico**
Countries following standard 50 µg/L	**Bangladesh, China, India, Indonesia**

Table 2. List of countries adopting different arsenic standard (Yamamura 2001).

2.6.6. Paradox nature of arsenic

There are many chemicals known to be carcinogen. The International Agency for Research on Cancer (IARC) has defined the list of agents which are well known and suspected human carcinogens. Arsenic has been classified as a class I human carcinogen by the International Agency of Research on Cancer [IARC, WHO], meaning that there is sufficient evidence of carcinogenicity to humans. Despite its potential to be carcinogenic, the information on molecular mechanism of action of arsenic is very limited till now (Martinez et al., 2011). But, on the other hand, arsenic is also known for many therapeutics applications since, earlier period. Arsenic is known to be very effective against treating various pathological conditions such as trypanosomiasis, syphilis, pernicious anemia, Hodgkin's diseases, eczema, asthma, psoriasis and currently it is used to treat relapse case of acute promyelocytic anemia (APL) (Evens et al., 2004). So, this dual nature of arsenic is enigmatic and very controversial as shown in **Fig.4**.

Although, arsenic is known to cause cancer in humans, but the mechanisms of action of arsenic in carcinogenesis is not well understood. On the other hand, its anti-cancer efficacy is well known but very little is known about its mechanism too. So, understanding this mechanism of arsenic is very important to resolve the enigmatic action of arsenic (Bode and Dong, 2002).

2.6.7. Arsenic toxicity and carcinogenesis

Arsenic is a well-known poison to human with many toxic effects on human health. The source of arsenic in environment could be through natural and anthropogenic resources. Human get exposure to arsenic through food, air and water. Arsenic exists in nature in

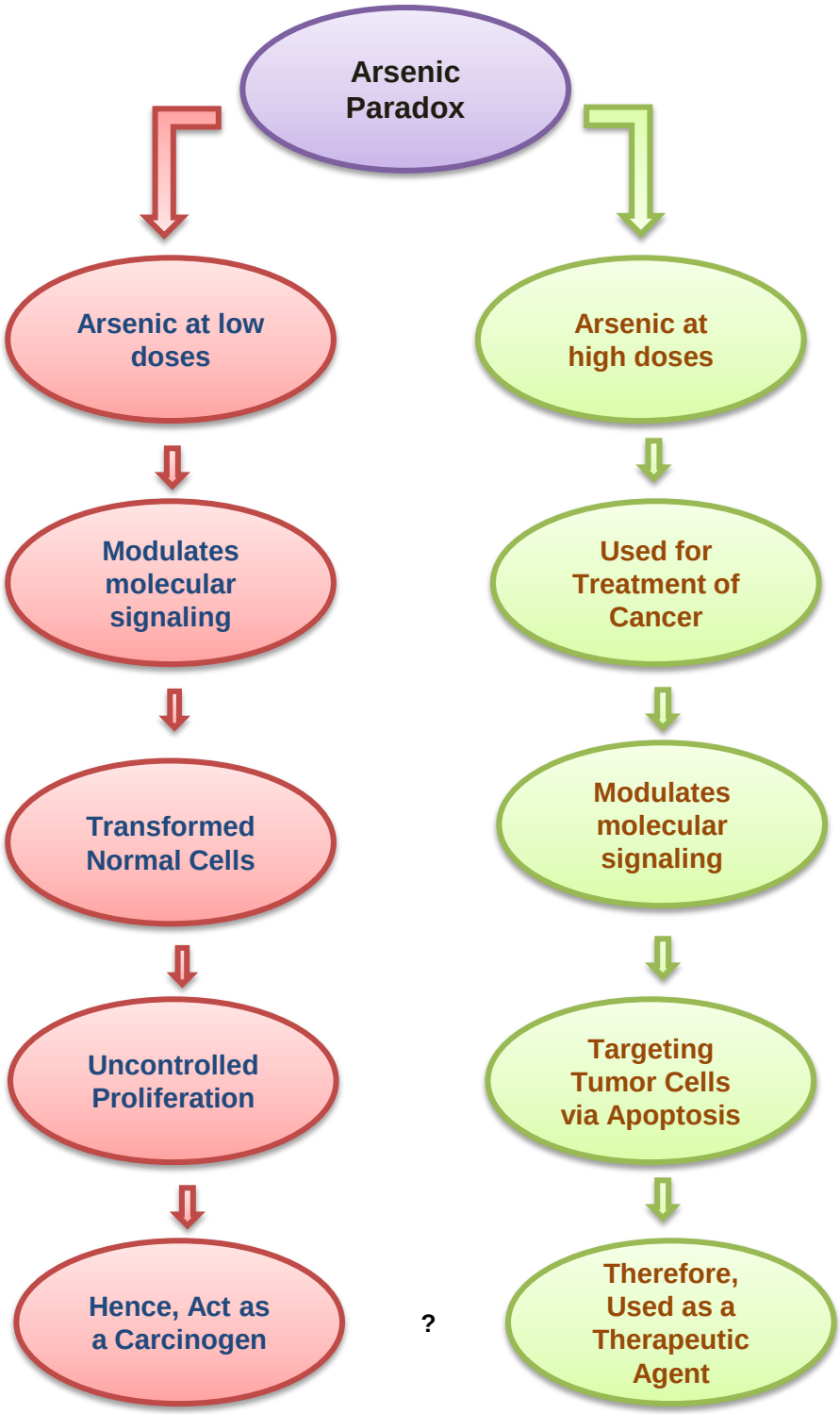


Figure 4. Flow–chart representation of the biphasic nature of arsenic. Low doses of arsenic cause transformation in HaCaT cells. However, arsenic is also prominently used in treating some cancers such as acute promyelocytic leukemia (APL).

different forms such as arsenite, arsenate, MMA and DMA. But the aspects of toxicity are different for each species of arsenic. As mentioned earlier, arsenite (As^{III}) is more toxic as compared to arsenate (As^{V}), however, the final and intermediate metabolites of arsenic have varied toxicity.

The toxicity of final metabolites of Arsenic such as monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}) is less while, the toxicity of intermediate metabolites such as monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) is more as compared to inorganic arsenic. So, the defined order of toxicity of arsenic species is As^{V} <MMA^{V} < DMA^{V} < As^{III}< MMA^{III} ≈ DMA^{III}. Arsenic is known to exert carcinogen effects in many organs such as liver, skin, bladder and lung. There are multiple mechanisms by which arsenic promote carcinogenesis, of which induction of ROS (Reactive Oxygen Species) is the most common which mediates arsenic related carcinogenesis. Arsenic is known to cause many other complications in the body other than cancer. Studies have shown that the arsenic exerts many epidemiology, cytotoxic and genotoxic effect on human and in many other animals as shown in **Fig.5** (Sun et al., 2014).

Studies have shown that arsenic generally does not induce point mutation but is known to cause deletion mutations in chromosome. Further, studies reported that arsenicals compounds causes genotoxic affects that were confirmed through micronucleus (MN) assay (Ren et al., 2011b)**.** The severity of toxic effect of arsenic depends on the age of the person, dose and genetic factors. Arsenic is also known to affect several enzymes in body (Jomova et al., 2011).

2.7. Pathological conditions associated with arsenic

Arsenic is known to cause many pathological conditions to human. The pathology associated with arsenic varies for acute and chronic exposure. The short term effects of arsenic can be observed within 30 minutes of ingestion, although delayed onset of symptoms has been observed if taken with food. The acute toxicities from arsenic include muscular pain, weakness, severe nausea, vomiting, abdominal pain and diarrhea. The tissue fragmentation can also be observed alongwith complains of numbness in hand and toes. The severity of symptoms depends on the extent of arsenic poisoning. In severe poisoning, there could be multiorgan failure that leads to death (Saha et al., 1999). According to WHO, the exposure of individual to arsenic rich water over a long period leads to arsenic poisoning known as arsenicosis. This poisoning affects the systematic function of many organs in the body. The most commonly affected and responsive organ is skin with clearly manifested

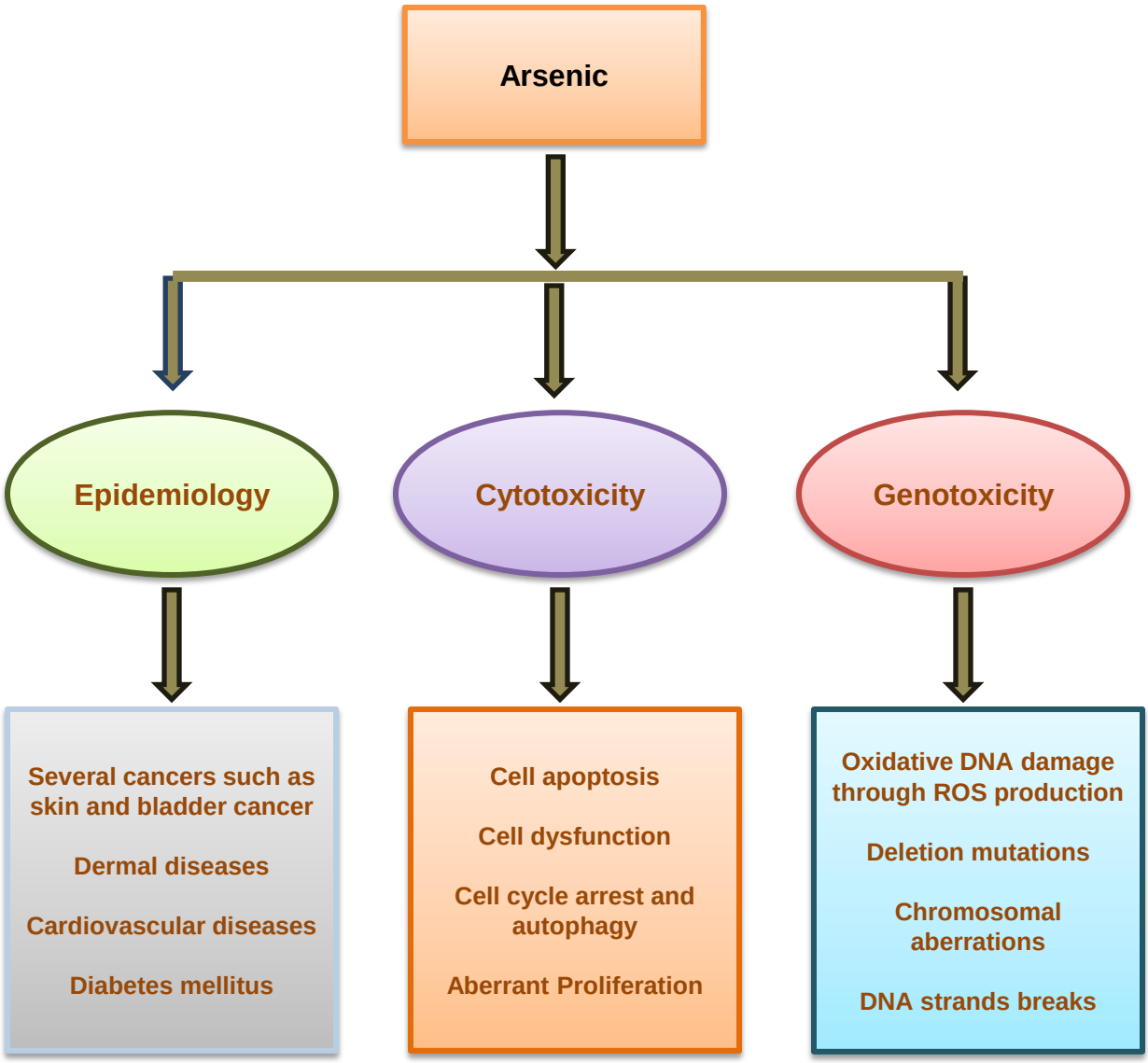


Figure 5. Schematic representation of deleterious effect of arsenic on humans as well as rats. Arsenic mediates its effect through multiple mechanisms. Arsenic causes cancer in multiple organs, predominantly in skin cells. It shows cytotoxic and genotoxic effect. Effects of arsenic is concentration dependent. At high doses, it causes cell apoptosis, ROS production and ODD (Oxidative DNA Damage) while at low dose it is non-cytotoxic to the cells, however, causes aberrant proliferation in cells.

pigmentation, but other parts also get affected from arsenic toxicity such as peripheral vascular system, kidney, heart, liver, lung, prostate, uterus, bladder and lymphatic tissues. Since, skin is the most sensitive organ to arensic toxicity, thus, the skin lesions is the most common symptom found in arsenic affected population. The extracutaneous effects of arsenic toxicity include atherosclerosis, hepatotoxicity (fibrosis), neurological, genitourinary and diabetes mellitus (Sengupta et al., 2008). The blackfoot disease, which is an endemic in Taiwan, is a disease of peripheral vascular system characterized by narrowing of peripheral vessels. The studies have shown dose-response relationship between arsenic and peripheral diseases (Tseng et al., 2007). The different stages featuring arsenic toxicity can be classified into four parts which is given by Dr. Saha and this classification is known as Saha's classification related to arsenic toxicity. These stages include **preclinical, clinical, internal complication and malignancy**.

The preclinical phase is asymptomatic which is further divided into two stages and these stages are blood phase and tissue phase. During blood phase, the arsenic content in blood and urine is found to be very high which upon drinking normal water can be reversed. The arsenic metabolites found in urine is DMA and trimethyl arsinic acid (TMAA). In tissue phase, high amount of arsenic is found in nails, hair and skin scales but no arsenic toxicity is observed. The clinical stage is defined by high concentration of arsenic in nails, hairs and skin scales but the symptoms observed in the individuals could vary. Dark pigmentation, melanosis and keratosis are the symptoms observed in clinical stage. In third stage i.e., internal complication, organs other than skin get affected. Organs such as lung, liver and spleen because of high arsenic accumulation become dysfunctional and hepatomegaly and splenomegaly is also observed. The last stage occurs after long period of time where malignancy occurs in lungs, skin, bladder and uterus (Saha et al., 1999). The pathologies associated with different organ system has been described as mention below.

2.7.1. Effect of arsenic on cardiovascular system

The effect of arsenic toxicity on cardiovascular system is well established. The arsenic poisoning lead to ventricular fibrillation in patient, alongwith a long QT interval is observed. The treatment of APL patients with arsenic trioxide (ATO) show prolonged QT interval that could be life threatening. The acute myocardial infarction is the leading cause of death when compared with lung and bladder cancer caused by arsenic poisoning. Several mechanisms of action of arsenic is known for these effects on cardiovascular system such as

stimulating ROS production, inhibitory effects on mitochondrial system, induction of proliferation of endothelial cells and lipid peroxidation (Jennrich, 2013).

2.7.2. Effect of arsenic on liver system

Liver is the one of the important organ of the human body. There are several reports published which have stated the correlation between chronic exposure of arsenic and liver dysfunction. In West Bengal, India, there are reports of hepatomegaly in patients affected with arsenicosis. Also, liver fibrosis and cirrhosis are another clinical manifested symptoms associated with chronic arsenic exposure (Liu and Waalkes, 2008). The arsenic accumulates in the liver since it is the most common site for arsenic metabolism. This tends to effect normal physiology of the liver. Also, it has been reported that the hepatic enzymes get elevated in blood with chronic exposure of arsenic (Saha et al., 1999).

2.7.3. Effect of arsenic on respiratory system

Arsenic is known to cause many non-cancer respiratory disorders. Many studies from different arsenic affected regions have shown that the cough is a prevalent disorder found in those drinking arsenic contaminated water. Studies from Chile have reported that the prevalence of bronchiectasis i.e. widening of bronchi, is increased by 23 fold in children exposed to arsenic water as compared to normal. The occupational exposure of arsenic is reported where inhalation of arsenic causes the respiratory symptoms such as lesions in mucous membrane, emphysema with decreased functionality of pulmonary system (Tchounwou et al., 2003).

2.7.4. Effect of arsenic on renal system

Kidney is another important organ involved in excretion which gets affected by arsenic. Kidney is the major site for the conversion of arsenate into more toxic and soluble arsenite form. Arsenic damages the integral part of the kidney such as glomeruli, tubules and capillaries which is essential for normal function of the kidney. The severity of damage depends on the uptake of arsenic as it can also induce hemolysis that could lead to acute tubular necrosis and thus, cause renal failure (Tchounwou et al., 2003).

2.7.5. Effect of arsenic on gastrointestinal (GI) system

The effect of arsenic on GI tract generally occurs when ingested and symptoms occur acutely after ingestion. Although, GI tract could also be affected if exposure is through some other routes. The symptoms include inflammation and necrosis of the mucosa and

submucosa layer of the stomach with lesions (ATSDR, 2011). The submucosal capillary damage marks the absorption of arsenic in gastrointestinal tissues. The vascular damage in the intestine leads to loss of fluid and proteins from the body. Nausea, vomiting, diarrhea and irritation of GI tract are some common symptoms that depends on the severity of the arsenic poisoning (Tchounwou et al., 2003).

2.7.6. Effect of arsenic on neuronal system

Arsenic induced neurotoxicity has been reported in both child and adult. It is reported that all form of arsenic such as inorganic or methylated form, get accumulated in the brain primarily in pituitary region. The chronic ingestion of arsenic affects a number of neurological and cognitive processes. Researchers have shown that arsenic affects the intelligence and learning related process and also mood disorders such as depression. Arsenic exerts its affects through modulating many signaling such as glucocorticoid signaling, cholinergic and monoaminergic signaling and alters the behavioral aspects such as locomotion, learning and memory (Tyler and Allan, 2014).

2.7.7. Effect of arsenic on development and reproductive system

The impact of arsenic toxicity on reproductive system and development is well established. Arsenic is a known teratogen which can cross the placenta easily and cause high rate of spontaneous abortions and malformations. Reports have published that acute arsenic ingestion at third trimester of pregnancy severely affects the infant and born infant dies within 12 hours due to alveolar hemorrhage and accumulation of arsenic in brain and liver. The malformations due to arsenic increase several fold when compared to normal (ATSDR, 2011). Placenta is very important for fetal development. Arsenic inhibits the thioredoxin reductase which protect the placenta from the oxidative stress which leads to damage to placenta. It leads to impairment of transportation of nutrients across placenta and thus, risks fetal life (Vahter, 2009).

2.8. Arsenic and cancer

Arsenic is well documented carcinogen affecting millions of people around the world. It is natural environmental contaminant which is ubiquitously present in the environment to which human is constantly exposed through air, food and water (Hughes et al., 2011a). Arsenicosis condition has been shown to be linked to various cancers such as liver, prostate, lung, skin cancer, etc. It is reported that risk of cancer increases in certain organs of the

body very significantly, notably lung, bladder, skin, liver and kidney, when exposed to arsenic (Mandal and Suzuki, 2002). The exposure to arsenic for many years leads to carcinogenesis in these organs.

The level of arsenic in blood falls to normal after few hours of arsenic intake but arsenic gets accumulated in others part of the body such as hair and nails. The epidemiological study of dose-response relationship of arsenic and cancer is limited by many factors such as exposure amount of arsenic, source of arsenic etc., but in few regions of Taiwan, the linear dose-response relationship has been found. The risk of cancer due to arsenic is affected by many others factors such as gender, genetic makeup of the individuals, coexistence of some other carcinogens in water, nutritional factor and smoking (Tapio and Grosche, 2006).

Arsenic related toxic events have been elucidated in many cell lines and animals models. The oxidative stress related DNA strand break due to arsenic is reported in human fetal lung fibroblast (2BS cells) and human alveolar epithelial type II cells (L-132 cells). The lung of mice when exposed to arsenic demonstrated decreased expression of gene transcripts involved in cell cycle, apoptosis, oxygen transport and lipid metabolism (Martinez et al., 2011). Several studies have reported that chronic exposure to arsenic at low dose (<100 μg/L) caused cancer of skin, lung, bladder and kidney while high dose (>250 μg/L) mostly associated with liver cancer. Arsenic and its metabolites mainly exert its effect through oxidative stress that leads to oxidative DNA damage (ODD). The Base Excision Repair (BER) pathway generally fixes the ODD. Thus, studies have shown the effect of arsenic and its metabolites on BER pathway in A549 lung cancer cells. One study have found that arsenite at dose 10 μM or more, attenuated DNA ligase III3a (LIG3) level while DMA^V at dose 5 μM decrease 8-oxoguanine DNA glycosylase-1 (OGG1) activity. Several other studies have also shown that the BER pathways get impaired by arsenic (Muenyi et al., 2015).

2.9. Arsenic and skin cancer

Skin carcinogenesis due to exposure to arsenic is a multistep process. Chronic Exposure to arsenic over a year causes many non-cancerous changes in skin that ultimately lead to skin cancer. Early clinical manifestations are changes in skin pigmentation which is followed by hyperkeratosis (Lindberg et al., 2008). There are many reports which have established the relationship between arsenic and skin cancer over past few decades. Arsenic has been known to cause three common malignancies in patients i.e. Bowen's disease, SCC and BCC. The appearance of malignancies caused by arsenic exposure varies from 10 year as in Bowen's diseases to 20 to 30 year latency period in other types of skin cancers.

The arsenic originated SCC either develops from *de novo* or progress from Bowen's disease. While the arsenic related BCC occurs at multiple foci in region that is not exposed to sunlight. The epidermal keratinocytes exposed to low arsenic exhibited differential expression of genes involved in molecular signaling involved in oxidative stress and modulation of MAPK (mitogen activated protein kinase) pathway (Martinez et al., 2011). Arsenic tends to accumulate in skin which leads to condition known as hyperkeratosis, a hallmark of the chronic exposure of arsenic. There are many clinical manifestations of chronic arsenic poisoning, most commonly observed symptoms include arsenical skin lesions, melanosis, conjunctivitis and keratosis (Basu et al., 2001).

Gender based effect of arsenic is also reported. Males are more susceptible in developing skin lesions when exposed to arsenic as compared to females. This susceptibility is because of differences in metabolism of arsenic in the body. There is high correlation between the methylation efficiency and risk of developing skin cancer in men and women. The population based study in Bangladesh have found higher amount of inorganic arsenic and methylarsonate (MA) while lower amount of DMA in urine of male, suggesting less efficient process of arsenic methylation among men. Several other studies are consistent with these studies of finding higher amount of inorganic arsenic in urine and chromosomal aberration in skin cancer and bladder cancer (Lindberg et al., 2008).

In developing countries, nutritional quality is another factor which renders the population more prone for diseases. Reports have shown that nutritional deficiencies increase the risk for skin lesions induced due to arsenic. The deficiency of folate, calcium, animal protein and fiber intake has been associated with increase skin lesions and cancer. Folate is known to be involved in methylation of inorganic arsenic. Therefore, studies have shown that the deficiency of folate has been associated with reduced arsenic methylation. But, supplement trial for folate have shown more arsenic methylation to DMA (Pilsner et al., 2009). Arsenite showed co-carcinogen effect with UV radiation on mice skin with more skin lesions found in combined arsenite and UV treatment as compared to alone (Burns et al., 2004). Association of exposure of inorganic arsenic through drinking water and skin cancer is not prominent in United States. There are others factors such as sunlight, deficiency of nutrition which act with arsenic and develops skin cancer. Skin cells are known to have low level of methylated arsenicals metabolites. DMA^V, the most common urinary metabolite, is known as a promoter of carcinogenesis in many organs but less is known about skin. Exposure of mice through drinking water with DMA^V at a dose 50 ppm for two year causes bladder cancer but no skin lesions were found. It suggests that arsenite but not its methylated

metabolites are carcinogenic species (Burns et al., 2004). Exposure of low dose of sodium arsenite to epidermal cells stimulate them to proliferate with overexpression of growth factors like keratinocytes growth factors, Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and transforming growth factor-α (TGF-α) and cytokine, tumor necrosis factor-α. The increase proliferation upon exposure with arsenic is indicated by increase in cell number, thymidine incorporation in DNA and c-myc expression (Germolec et al., 1996).

2.10. Arsenic as an anticancer agent

Arsenic is known for its medical applications for long time in treating many diseases including cancer. The therapeutic efficacy of arsenic as a drug for effectively treating APL is well established. Also, arsenic trioxide and related derivatives are being tested for wide range of hematological malignancies like myelodysplastic syndromes, chronic myelogenous leukemia and multiple myeloma (Ralph, 2008). The studies have shown that arsenic trioxide (As_2O_3) mediates its effect through mitochondria based ROS generation. The As_2O_3 interfered with the electron transport chain (ETC) in leukemia cells, thus enhanced ROS production and induced apoptosis in these cells. There are many evidences that suggest that mitochondrion is involved in As_2O_3 mediated cytotoxicity. Firstly, the incubation of cells with drug for 3 hours causes substantial inhibition of mitochondrial respiration and increased ROS production, which is observed earlier than apoptosis. Secondly, there was no significant increase in ROS production and apoptosis observed when respiratory deficient cells, C6F/ρ^-, was treated with arsenic trioxide. It suggests that ATO mediates its cytotoxicity through mitochondrion (Pelicano et al., 2003). The arsenic trioxide induced the release of cytochrome c from mitochondrion membrane and subsequent, activation of caspase-3 and induced apoptosis.

Furthermore, ATO elevated the calcium concentration in the cells and thus, disturbed the calcium homeostasis within the cells. Thus, the arsenic induced cytotoxicity due to disruption of calcium signal could be one important factor but other mechanisms could also be involved (Florea and Busselberg, 2009). The studies on the effect of ATO on APL cell lines have shown that it induced the activation of caspases and downregulated Bcl-2 and upregulated p53 protein expression. The ATO effects the transcription of several genes involved in mitogenic signaling, apoptosis and cell cycle progression in HL-60, promyelocytic leukemia cells. The preclinical studies have reported increased synergistic effect of ATO and ascorbic acid against HL-60 cells in combined as compared to alone

(Yedjou et al., 2010). Most of the cases of APL express PML-RARα fusion protein, which resulted from reciprocal translocation of their gene. The drug all-trans retinoic acid (ATRA) is used for treating APL where nearly all patients undergo remission. ATO mediated differentiation of granulocytes is achieved by complete degradation of PML-RARα fusion protein. However, the mode of action ATO is more complex and depends on many factors such as cellular environment, dose and cell type (Jing, 2004; Zhang et al., 2001). ATO could be used effectively in treatment of cancer of cervix, ovary, prostate and neuroblastoma (Florea and Büsselberg, 2013).

2.11. Metabolism of arsenic

Ingestion of arsenic over a period of time is critical factor for developing various types of cancers. Arsenic is mainly found in arsenite or arsenate form in drinking water. Humans are exposed to arsenic mainly through drinking water. The most common form of arsenic in drinking water i.e., arsenite and arsenate are reported to show 80-90% absorption in gastrointestinal tract in human and experimental models of animal (Freeman et al., 1995; Pomroy et al., 1980; Vahter and Norin, 1980). The less soluble form of arsenic, for example, arsenic trisulfide and lead arsenate (Marafante et al., 1985), arsenic selenide (Mappes, 1977) and gallium arsenide (Webb et al., 1984) is absorbed less efficiently in tract. The studies on the rate of absorption of arsenic through skin have not been explored much, but incidence of arsenic toxicity is known to persons on having acute exposure of inorganic arsenic solution on skin. *In vitro* studies on mice dermal skin have shown that 30% of the applied radiolabeled arsenate in solution was absorbed within 24 hour. But, 60-90% of applied solution retained in the skin (Rahman et al., 1994). The indirect evidence for absorption of arsenic through skin is supported from studies in Alaska where the people used arsenic contaminated (around 345 μg/L) water for washing, but for drinking arsenic free water was used. It was found that these topical exposed people have same low level of arsenic metabolites in their urine as people who were exposed to arsenic through drinking water (Harrington et al., 1978).

The absorbed arsenic gets diffuse in the circulatory system where it binds with several proteins mainly those containing sulfhydryl (SH) group and glutathione (GSH). Inorganic form of arsenic is found to be bound with serum proteins predominantly (Zhang et al., 1997). Studies have reported that a fraction of arsenic binds to transferrin protein also. Some species accumulate arsenic in red blood cells where it predominantly binds to hemoglobin. Majority of arsenic present in human blood is cleared within few hours after

absorption. Studies have reported that the half-life of arsenic in blood is about 1 hour. The binding of arsenic to hemoglobin is species specific as rat shows higher affinity as compared to humans (Shen et al., 2013). Radiolabeled arsenic has been used to detect the distribution of arsenic in mice. The intravenously administration of radiolabeled As^{III} and As^{V} in mice have resulted in level of high whole body concentration and retention of arsenic in mice having As^{III} injection as compared to mice having As^{V} injection. Kidney and skeletal muscles have shown higher level of As^{V} mice as compared to As^{III} mice, in skeletal muscle probably due to resemblance of arsenate to phosphate. The long duration binding of arsenic in hair, skin and upper GI tract attributed to high binding affinity of arsenic to keratin (Lindgren et al., 1982). Thus, arsenic tends to accumulate in those tissues or organs which have high content of cysteine rich proteins. Studies in West Bengal and Bangladesh have shown that exposure to high arsenic concentration through water leads to high accumulation of arsenic in hair, skin and nail which exceeds 40 mg/kg (Arsenic in drinking-water, WHO).

The reports of autopsy from suicide case from arsenic have shown that the acute intoxication with arsenic have shown highest accumulation of arsenic in kidney and lungs but the concentration of arsenic found to be 7 to 350 fold less in blood when compared to other organs. The As^{III} is more predominant form while As^{V} found in smaller quantities in lung, kidney and blood. The muscles and heart are the organs demonstrating high accumulation of arsenic besides liver and kidney. *In vivo* studies in rabbits have illustrated the presence of arsenic in 11.6%, 1.4%, and 1.7% of dose in liver, kidney and lungs after 5 hour of administration of arsenic (Benramdane et al., 1999).

In mammals, inorganic arsenic gets methylated to less toxic metabolites MMA and DMA which is easily excreted from the urine. But there are differences in methylation process that varies among species. Rodents and dogs methylate arsenic more efficiently as compare to humans. Also, they excrete arsenic fast as compared to humans. On the other hand, the monkey and the chimpanzee are the only mammals which do not methylate inorganic arsenic due to extensive binding of arsenic to tissue. Variation of arsenic metabolism among species is not much known but animal studies have shown that there are several factors which effect the process of methylation. These factors include dose of arsenic, route of administration, species of arsenic and nutritional status. Reports have found varying susceptibility to arsenic among individual when exposed to specific dose of arsenic. Children have less methylated efficiency as compared to adult. Also, men showed lower methylation rate when compared to women (Vahter, 1999; Vahter et al., 1995).

The methylation of arsenic is a sequential process in which As^{V} gets reduced into As^{III} and subsequently methyl groups are attached to the reduced form. The formation of trimethylated metabolites from inorganic arsenic takes place in few microorganisms. In human, the studies have shown that when given single oral dose of DMA of 0.1 mg/kg, individuals excreted 3.5% of this dose as trimethylarsine oxide (TMAO) in urine. The TMAO was found to induce mitotic arrest and tetraploids conditions in mammalian cell lines (Kenyon and Hughes, 2001). The metabolic product, MMA^{V} and DMA^{V}, are the major inorganic arsenic metabolites along with MMA^{III} and DMA^{III} found in human urine (Hayakawa et al., 2005). The reports have concluded that the inorganic pentavalent arsenic, dimethylmonothioarsinic acid ($DMMTA^{V}$) are very toxic metabolite of arsenic. It is reported that some species lacks the arsenic methylation capacity but their susceptibility to acute arsenic is comparable to other animals that possess the methylation capacity. Thus, the process of methylations of inorganic arsenic is not necessarily the complete detoxifying process (Watanabe and Hirano, 2013).

The mechanism of arsenic biotransformation in living system is not well defined, although two mechanisms have been proposed to describe this important process**.** The generally accepted mechanism of arsenic biotransformation involves many oxidations and reductions that are coupled with methylations (Aposhian and Aposhian, 2006). Another reasonable mechanism involves the formation of arsenic and glutathione complex which might occur due to action of enzymes and this pathway is known glutathione conjugation or reductive methylation pathway. The formation of trivalent arsenic and GSH complex, $As^{III}(SG)_{3,}$ is very significant to the pathway. The complex, $As^{III}(SG)_{3,}$ acts as substrate for the enzyme Arsenic (+3 oxidation state) methyltransferase (AS3MT) (Watanabe and Hirano, 2013).

The conversion of inorganic arsenic into MMA^{V} and DMA^{V} requires an enzyme AS3MT that found in number of species like rat, mouse, rabbit and monkey including humans. The studies have shown that in rat AS3MT, which has cloned from liver cytosol, requires an S-adenosylmethionine (SAM) for their activity. The studies from Bangladesh, West Bengal and Mexico have reported polymorphism of AS3MT in human (Watanabe and Hirano, 2013). The AS3MT knock out study in mice have shown when these mice exposed to arsenate, their urine and liver samples found to have mono- or dimethylated arsenicals when compared to wild type, but to lesser extent. This suggests that there could be alternative pathways involved in methylation of arsenic. The studies have reported that another enzyme N6AMT (N-6 adenine-specific DNA methyltransferase 1) is specifically involved in the biomethylation of MMA^{III} to DMA (Ren et al., 2011a).

2.12. Transporters of arsenic

The transport of arsenic across cell membrane is important for arsenic to mediate its toxic effects through metabolism as shown in **Fig.6**. There are many arsenic transporters have been reported in prokaryotes as well as in eukaryotes including mammals. The family of major intrinsic proteins (MIP) is composed of transmembrane proteins involved in transport of water, urea, ammonia, carbon dioxide and glycerol in energy independent manner found in both eukaryotes as well as prokaryotes.

In prokaryote, the studies in *E.coli* have shown that two phosphate transporters, Pit and Pst, are involved in arsenate uptake (Rosen, 2002), (Yang et al., 2012). GlpF is a glycerol transporter found in E.coli, first identified member of MIP superfamily, when mutated leads to significantly reduction of arsenic uptake in bacterial cells (Yang et al., 2012). Various phosphate transporters have been identified in yeast such as Pho84, Pho89, Pho87, Pho90 and Pho9 which is involved in the transport of arsenate. Studies have shown that the deletion of Phos84 and Phos87 genes resulted in increase of arsenic tolerance in yeast, which strongly suggest the association of phosphate transporter with arsenic ($As^{V)}$ uptake (Maciaszczyk-Dziubinska et al., 2012). Subsequently, the conversion of As^{V} to As^{III} takes place in cells that is catalyzed by arsenate reductase Acr2p.

The transportation of arsenic from cells is bidirectional as efflux of As^{III} from cells also takes place that is mediated by Fps1p and arsenite permease, Acr3p (Yang et al., 2012). Because of similarity between As^{V} and inorganic phosphate (Pi), it is assumed that As^{V} cross the membrane using Pi transporter. Transportation of arsenic through sodium/phosphate cotransporter type II (NaPiIIb) has been reported in *Xenopus laevis* oocytes (Villa-Bellosta and Sorribas, 2010). It has been found that in *S. cerevisiae,* the glycerol transporter Fps1, a MIP protein, was also shown to involve in transportation of As (III) (Maciaszczyk-Dziubinska et al., 2012).

Aquaglyceroporins transport small uncharged molecules such as glycerol, ammonia and urea along with water. In mammals, many types of aquaglyceroporins are found. AQP7 and AQP9 are two members of aquaglyceroporins which are found majorly in kidney and liver, respectively. Both transporters are involved in the uptake of As^{III} (Liu, 2010). *In vivo* studies in AQP9 null mice have shown that AQP9 plays a pivotal role in excretion of arsenic and thus prevent arsenic toxicity. These mice were unable to excrete arsenicals through urine and thus many tissues was found to have very high arsenic level (Yang et al., 2012). The ATP binding cassettes (ABC) proteins are the family of transporter which are also involved in arsenic transportation across membrane. The multidrug resistance protein 1

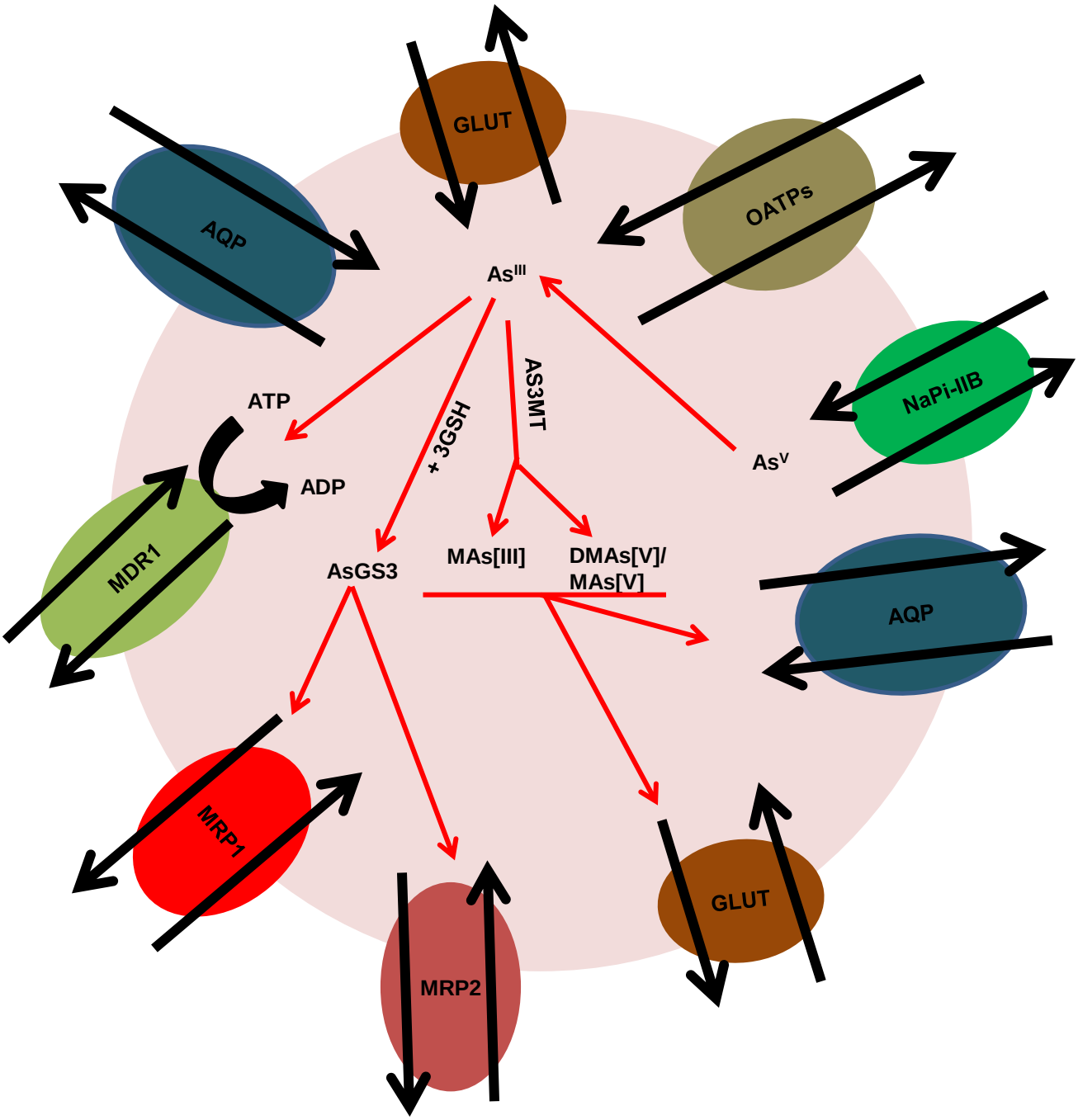


Figure 6. Schematic representation of mammalian transporters involved in influx and efflux of arsenic across membrane. Many transporters such as glucose transporters (GLUTs), phosphate transporters (Pi), aquaglyceroporins (AQPs), organic anion transporting polypeptides (OATP) and ABC transporters such as multidrug resistance-associated protein family (MRP) and multi-drug resistance (MDR) are involved in bidirectional movement of arsenic across membrane.

(MRP1) is an ATP transporter protein involved in transportation for arsenite conjugate complex thus, protect the cells from the arsenicals toxicity (Carew et al., 2011). Studies have illustrated the role of glucose transporters (GLUT) and organic anion transporting polypeptides (OATPC) in arsenic absorption from intestine. Caco-2 cell line is an appropriate model to study intestinal transportation as it expresses many transporters of intestine. These cells when treated with 10 µM As^{III} lead to increase in expression of many transporters such as GLUT2, GLUT5 and AQP4 (Calatayud et al., 2012).

2.13. Mechanisms of action of arsenic

Arsenic mediates its effect on transformation of cell through various modes of action. The mechanism of actions of arsenic is interdependent, through various biochemical effects of arsenic.

2.13.1. Interaction of arsenic with sulfur and phosphate

As previously mentioned that the arsenite interact with a sulfhydryl group very effectively. There are certain significant enzymes such as pyruvate dehydrogenase (PDH) which are highly sensitive to arsenic as it contains vicinal dithiols. This enzyme is inactivated through the binding of arsenite to its sulfhydryl group, but other studies have shown that generation of ROS through arsenite is responsible for the inactivation of this enzyme (Hughes et al., 2011a).

Because of similarity in structure as well as physiochemical properties of arsenate and phosphate, the arsenate interferes with several reactions of phosphate within a cell. *In vitro* studies have shown the uncoupling of formation of adenosine-5'-triphosphate (ATP) process by arsenate through mechanism known as arsenolysis. Human RBCs dies in few hours after exposure to arsenic and it might be due to the ATP depletion and loss of membrane integrity (Hughes et al., 2011a).

2.13.2. Formation of ROS

The metabolism of arsenic mainly occurs in liver, and during this process the generation of ROS and reactive nitrogen species (RNS) is very common. Most of the free radicles formed in the cells includes superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH), collectively known as ROS. These molecules are formed in the cell through normal metabolic processes such as mitochondrial respiration. Normally, cells have mechanisms to remove these reactive species but formation of these in excess amount is

toxic to the cells. The reactive species are formed *in vitro* as well as *in vivo* in the presence of arsenite. These reactive species formed includes superoxide anion, hydroxyl radical, hydrogen peroxide, reactive nitrogen species and arsenic peroxyl radicals (Kitchin, 2001). In several studies, it is shown that addition of antioxidants and radical scavengers decreases the arsenic-induced ROS formation and their related toxicity (Shi et al., 2004). However, the mechanism of ROS formation by arsenic is not clear. It is considered that ROS are formed during the conversion of arsenate into arsenite, by the formation of intermediate arsine during arsenite metabolism and stimulation of Nicotinamide adenine dinucleotide (NADH) and NADPH oxidase (Lynn et al., 2000).

Most of the actions of arsenic are directly or indirectly related to formation of ROS. It has been reported that arsenic mutagenicity is mediated by certain species of ROS, especially hydroxyl radicals. The studies consistent with this have shown that the antioxidant enzymes such as glutathione peroxidase, superoxide dismutase (SOD) and catalase diminish the deleterious effect of arsenic, such as chromosomal aberrations and exchange of sister chromatids, in mammalian cell cultures (Hei et al., 1998). The increase risk of developing cancer in human has been well established with oxidative stress induced by arsenic. However, the exact mechanism of formation of ROS has not defined yet, although formation of many free radicles is known mainly hydroxyl radicals. The role of mitochondrion and ATP depletion in arsenic induced toxicity is prominent. Arsenite is also known to decline the mitochondrion membrane potential (Pourahmad et al., 2005). The perturbances in ROS could affect the activity of transcription factors involved in maintaining homeostasis of cell, thus may promote risk of mutation and carcinogenesis.

2.13.3. Genotoxicity caused by arsenic

Several studies has been shown that arsenic never interacts with DNA directly to cause mutation although *in vitro* studies have shown the interaction of arsenic with DNA and introduction of mutation (Labuda et al., 2005). It is also known that arsenic has many genotoxic effects such as mutations, oxidative DNA damage, DNA strand breaks, sister chromatid exchanges, chromosomal aberrations, aneuploidy, and micronuclei formation. Other effects of arsenic include gene amplification, transforming activity and genomic instability which has been observed *in vitro* as well as *in vivo* (Hughes et al., 2011b). Arsenic is also known to act as a co-mutagenic agent. It enhances the mutagenic effects of UV radiation in bacterial and mammalian cells (Chen et al., 2005) as well as of mutagenic agents such as methyl methansulfonate and N-methyl-N-nitrosourea in mammalian cells.

2.13.4. DNA repair alteration caused by arsenic

DNA damage or lesions in the DNA triggers common signaling pathways collectively referred as DNA damage response (DDR). DDR either slows or arrest cell cycle, which is important for DNA repair, before proceeding to DNA replication. The study has shown that chronic exposure of arsenite at dose 0.1 µM in HaCaT cells resulted in poly (ADP-ribosyl)ation of TP53 and poly (ADP-ribose) polymerase 1 (PARP1). The DDR is mediated by activated p53 which induces the transcription of p21 (cyclin dependent kinase inhibitor 1A, CDKN1A) and causes cell cycle arrest. By doing so, DNA damage repair system repair the damage before continuing the cell cycle. However, the arsenite has been reported to inhibit the PARP1 and decrease the expression of p21 at mRNA as well as protein level in HaCaT cells. In addition, the arsenite also inhibits the base excision repair (BER) and nucleotide excision repair (NER) pathways (Muenyi et al., 2015). One of the study has reported that individuals exposed to arsenic have decreased expression of genes associated with NER pathway, thus impairing the DNA repair mechanism. These downregulated genes are critical for the DNA incision and helicase activities (Andrew et al., 2003).

2.13.5. Effect of arsenic on DNA methylation

DNA methylation at promoters is involved in regulation of gene expression. Arsenic affects the expression of many genes by altering the DNA methylation. Studies have shown that human exposed to high levels of arsenic are significantly more likely to have p16 DNA hyper methylation (Lu et al., 2014). In another study, the hypermethylation of a portion of the p53 promoter region is observed in human lung adenocarcinoma A549 cells when treated with arsenite. It has also been observed that genomic DNA hypomethylation in human prostate epithelial cell line following a long-term exposure to arsenite resulted in malignant transformation of the cells (Hughes et al., 2011a). Long term exposure of low dose of arsenic affected the epigenetic regulation of SIRT1 expression through methylation in human keratinocytes which may lead to skin cancer (Herbert et al., 2014).

2.13.6. Effect of arsenic on signal transduction

Arsenic is known to effects many signal transduction pathways involved in apoptosis, proliferation, and differentiation of cells. Studies in endothelial cells from vasculature or liver sinusoidal have shown that the arsenite binds to sphingosine-1-phosphate receptor (S1P1), type of G- protein couple receptor (GPRC), and stimulates the receptor. This

stimulation causes the activation of Rac1 GTPase activity. Thus, arsenite binds to extracellular receptor and effect the cellular physiological response (Druwe and Vaillancourt, 2010). Arsenite activates multiple MAPK (Mitogen-Activated Protein Kinase) pathways, extracellular-regulated protein kinases (ERKs) and JNK (c-Jun N-terminal kinase) pathway. The activation of JNK through arsenite or arsenate requires Rho and Rac (Porter et al., 1999) (Hughes et al., 2011a). Arsenite is known to induce the expression of proto-oncogenes *c-jun* and *c-fos* in HeLa cells through activation of JNK and p38. Arsenite also affects the EGFR signal pathway and several transcription factors such as nuclear factor- κβ (NF- κβ) and nuclear factor erythroid-2-related factor 2 (Nrf2) (Hughes et al., 2011a).

2.14. *In vivo* models for arsenic study

Various studies have been done to examine the effect of inorganic arsenic in animal models for its carcinogenic effect. However, the induction of cancer due to inorganic arsenic in animal model is rare but the organic metabolites of arsenic such as DMA has been shown to be tumorigenic in animal models. Studies have shown the association between arsenic exposure and angiogenesis in animal model. The exposure of mice with arsenic at a dose of 10 ppb enhanced angiogenesis, and longer exposure time accompanied the recruitment of inflammatory cells (Straub et al., 2007). The effect of DMA, given in drinking water for 80 weeks, was studied in heterozygous (p53+/-) mice and wild type C57BL/6. The wild type showed spontaneous tumors while earlier induction of tumors was observed in knockout mice. The effect of organic arsenic as a promoter in animals is widely observed (Wang et al., 2002). The tumor suppressor genes, $p16^{INK4a}$ and RASSF1A, undergo epigenetic modifications in mice exposed to arsenic. The histopathology and RNA of lungs removed from A/J strain of mice expose to arsenate at different dose for 18 week was examined. It was observed that arsenate caused hyper methylation of these tumor suppressor genes resulted in reduced or loss of activity. Moreover, the number of tumor observed in treated mice was more as compared to normal (Cui et al., 2006). Also, the studies of effect of transplacental exposure to arsenic in animals have been done. The maternal exposure to arsenite through consumption of drinking water during gestation period found to be carcinogenic in offspring in several organs such as liver, adrenal and ovary. In various studies, arsenic is known to enhance the effect the other carcinogen. Arsenic acts as the promoter of the renal tumor in rats when given oral arsenite or DMA^V (Tokar et al., 2010). In another study, K6/ODC mice were given DMA^V and arsenate orally have shown positive

although low carcinogenic effect in skin that provides insufficient information, hence difficult to interpret. The treatment of K6/ODC mice topically with 7,12-dimethylbenz[α]anthracene (DMBA) followed by DMA^V applied to same skin causes more skin tumor multiplicity when compare to mice treated with DMBA only. Hence, arsenic acts as co-carcinogen and increase risk for developing skin tumor when given with other carcinogen in animal models (Tokar et al., 2010).

2.15. Cancer chemoprevention

"Cancer chemoprevention involves the chronic administration of a synthetic, natural or biological agent to reduce or delay the occurrence of malignancy" (Steward and Brown, 2013). The global burden of cancer is increasing every year which is causing many deaths all over the world. The illness and cost of the treatment of cancer causes distress to the family, so there is a need to find more new strategies to combat this disease. In past few decades, worldwide research have found that the chemopreventive agents are very effective against many cancers, which either suppress the early carcinogenesis or prevent the progression of premalignant cancer into malignant.

Chemoprevention can be classified into three types that are based on time of administration of chemopreventive agents. In primary chemoprevention, healthy individuals are given chemopreventive agents, particularly to those who have more risk factors. Secondary chemoprevention is given to the individuals who have premalignant lesions and thus prevent the progression into malignant. The individuals who have already gone under treatment of cancer are given tertiary chemoprevention, where these agents prevent the recurrence of cancer (Steward and Brown, 2013). Chemotherapy usually disrupts or delays the multiple pathways and process involve in initiation, promotion and progression of cancer**.** These agents can be classified as blocking agents or suppressing agents depending upon their mechanism of action. Blocking agents acts at initiation step of carcinogenesis and prevents the DNA damage by deactivating the carcinogens while suppressing agents acts at later stages of carcinogenesis i.e., effects promotion and progression, inhibiting the proliferation of initiated cells. Chemopreventive role of dietary and non-dietary phytochemicals are clearly shown in many cancers. Dietary phytochemicals including apigenin, curcumin, resveratrol and synthetic drugs including non-steroidal anti-inflammatory drugs (NSAIDs) and aromatase inhibitors are already known to have anti-cancer efficacy. These phytochemicals affects the several pathways such as JAK signaling,

NF-KB signaling, PI3K signaling, ERK signaling and redox signaling (Landis-Piwowar and Iyer, 2014).

2.15.1. Skin cancer and chemoprevention

The use of phytochemicals and other natural and synthetic compounds in skin cancer chemoprevention is an appealing approach since these agents are nontoxic, widely available and less toxic as compare to chemotherapy. Skin carcinogenesis is a multistep process, involving many molecules and signaling pathways as shown in **Fig.7**, thus allow many targets for chemoprevention. The sources of phytochemicals are diverse in nature such as vegetables, seeds, flowers, leaves and barks and these are the reservoirs of many active compounds such as flavonoids, isoflavonoids, phytoalexins, anthocyanidins, polyphenols, proanthocyanidins and carotenoids. Researchers have shown the protective role of these phytochemicals against skin cancer.

In one study, the dietary feeding of phytochemical proanthocyanidins extracted from grapes *(Vitis vinifera)* to SKH-1 hairless mice have shown to reduced the effect of UVB induced photocarcinogesis in skin at all stages of the skin cancer. Apart from inhibiting initiating activities of UV exposure, the extract also inhibited the UVB induced global DNA hypomethylation in mice skin. In SKH-1 hairless mice, the malignant conversion of skin benign tumor is due to UV induced reactive species which causes the genetic and epigenetic changes in the tumor cells. The extract is shown to be very effective against UV induced oxidative stress, thus inhibit the conversion of benign skin papilloma into malignant (Mittal et al., 2003). The oral feeding or topical application of green tea polyphenols (GTP) in female SKH-1 hairless mice had significantly reduced the UVB induced photo-carcinogenesis, although the effect of topical exposure was lower as compared to oral feeding. The UVB exposure to mice skin caused cutaneous edema resulting in depletion of antioxidant system and induction of activities of enzymes such as ornithine decarboxylase (ODC) and cyclooxygenase (COX). The oral feeding to mice with 0.2% GTP (w/v) only in drinking water for 30 days provides significant protective effect against depletion of antioxidant system and induced ODC and COX activities (Wang et al., 1991) (Agarwal et al., 1993). The topical application of epigallocatechin gallate (EGCG) provides photoprotection and prevents the UVB induced depletion of antioxidant enzymes. Moreover, the topical treatment of mice skin with ECGC inhibited the UVB induced phosphorylation of ERK1/2, JNK and p38 proteins (Singh et al., 2014). Recently published case study of a 72 year old man, who had been occupational exposed to arsenic through dust

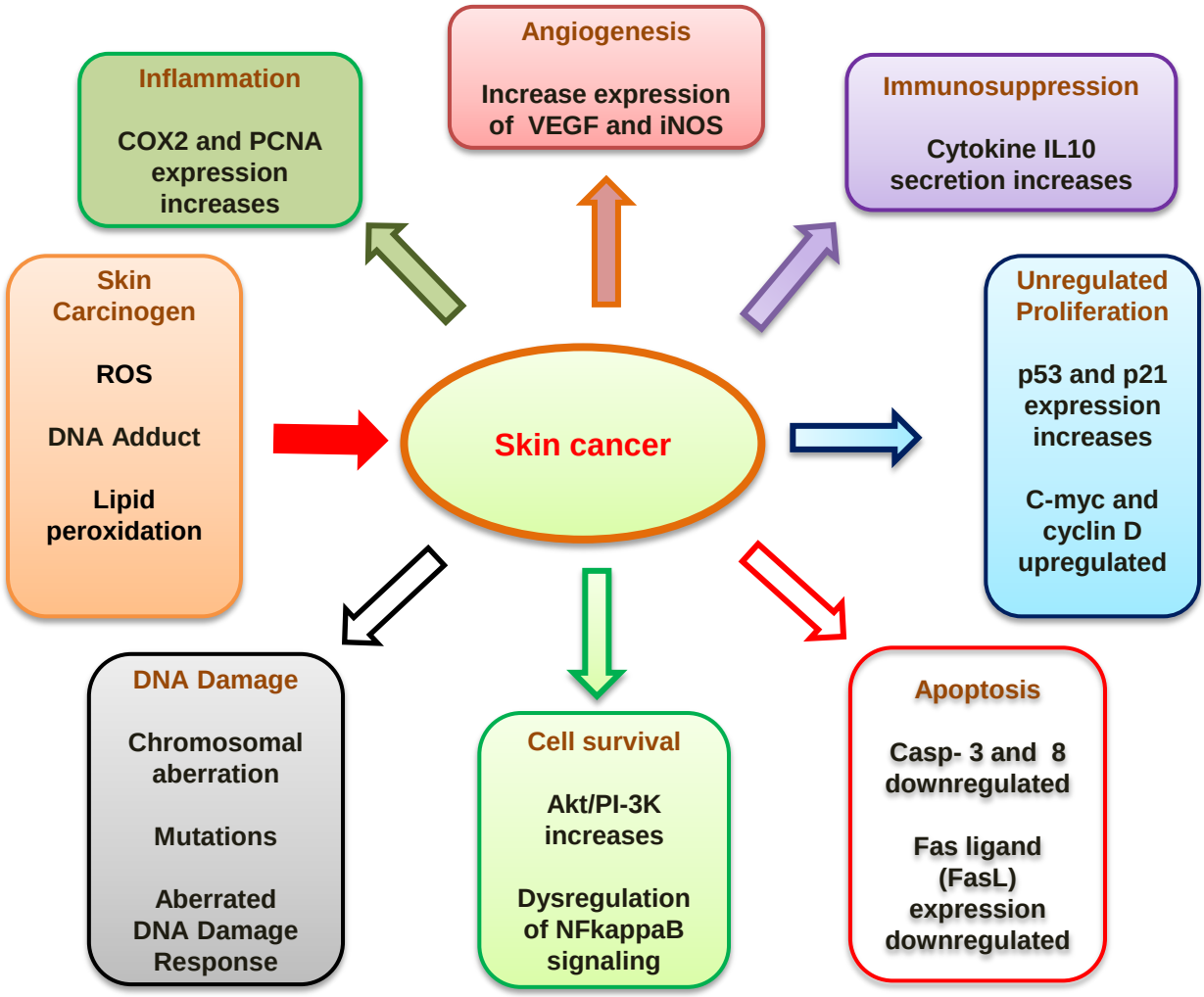


Figure 7. Molecular alterations involved in skin carcinogenesis have been shown in the diagram which provides many targets for chemoprevention. Skin carcinogenesis is a complex process that is accompanied by several molecular alterations. Major physiological processes involved in skin carcinogenesis include DNA damage, apoptosis, cell survival, angiogenesis, inflammation and increased proliferation. Many important molecules, related to these physiological processes, expression and activity get affected during the process such as caspase-3, 8, cyclin D, p53, p21, VEGF, COX2 etc. These molecule alterations provides many therapeutics target used in chemoprevention.

and skin contact for 3 years in his late fourties, had multiple lesions in the skin. To reduce the burden of skin lesions and prevent the conversion of these lesions into malignant form, the patient was treated with acitretin, a retinoid, at a dose of 20 mg/day for 9 months. The treatment was well tolerated and follow up for 5 years with no malignancy was documented (Wollina, 2016). There are many other known phytochemicals which are very effective against skin cancer chemoprevention.

2.15.2. Role of phytochemicals, silibinin and fisetin, in cancer

Silibinin

Silibinin (mol. wt. 482.44.) is the major biological active constituent of silymarin isolated from dried fruits and seeds of *Silybum marianum* (milk thistle) as shown in **Fig.8.** Silibinin is a naturally occurring flavonolignan with long history of its use. The milk thistle extract (MTE) has been used for hepatic disorders and against mushroom poisoning since long time. It has shown to be very effective against many cancers such as skin, lung, colorectal and prostate cancer (Mateen et al., 2013).

Silibinin is reported to protect the SKH-1 hairless mouse skin from UVB radiation. Silibinin protect the mice from UVB induced DNA damage and further induce the p53/p21 cascade to prevent the propagation of UVB mediated cell proliferation and apoptotic sunburn cells (Dhanalakshmi et al., 2004). Reports have shown the anti-proliferative and apoptotic effects of silibinin on colon carcinoma cells, HT-29. This effect of silibinin is associated with several molecular alterations in cancer cell such as increase in p27 and p21 at both mRNA as well as protein level and strongly inhibits the CDK2 and CDK4 kinase activity (Agarwal et al., 2003). Prostate cancer (PCA) is one of the leading cause of cancer deaths in american males because of its high invasiveness. Silibinin has shown to inhibit the PCA cells of origin in different species such as rats, mice and human, alongwith the human PCA cells xenograft in nude mice. There are several molecules and pathways that silibinin targets in PCA such as insulin-like growth factor-1 receptor (IGF-1R), EGFR and NF-κB pathways (Singh and Agarwal, 2006).

The synergistic studies of silibinin with other drugs have shown enhance efficacy of other drugs as compare to alone. In one such study, the silibinin strongly synergized the effect of doxorubicin in prostate cancer cells. There was stronger G2-M cell cycle arrest in combined treatment than silibinin and doxorubicin alone treatment which was associated with strong inhibition of cdc25C, cdc2/p34 and cyclin B1 expression and also cdc2/p34 kinase activity

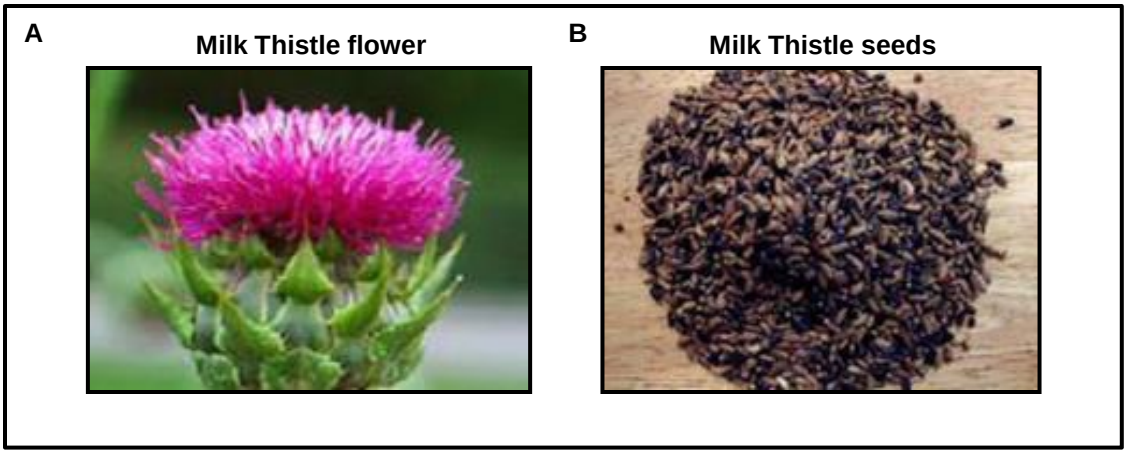
(Tyagi et al., 2002). Pancreatic cancer cell lines, AsPC-1, BxPC-3 and Panc-1 have shown growth inhibition and apoptosis when treated with silibinin. Silibinin activated the cascades of caspase-3, 8, 9 and thus induced apoptosis in pancreatic cancers cells and also induce G1-phase cell cycle arrest but only in AsPC-1 cells (Ge et al., 2011).

Silibinin is effective against both small cell lung cancer, SCLC (SHP-77) and non-small cell lung cancer, NSCLC (A549) cells. Silibinin cause growth inhibition, cell cycle arrest and apoptosis in these cancer cell lines in dose and time dependent manner. Several other types of NSCLC cell lines such as H1299, H460 and H322 are also sensitive to the silibinin (Mateen et al., 2013). Overall, the phytochemical silibinin have shown very promising results against several cancers while being nontoxic to the normal cells. Hence, exploring silibinin and other phytochemicals mechanism in detail could be provide new insights in cancer research.

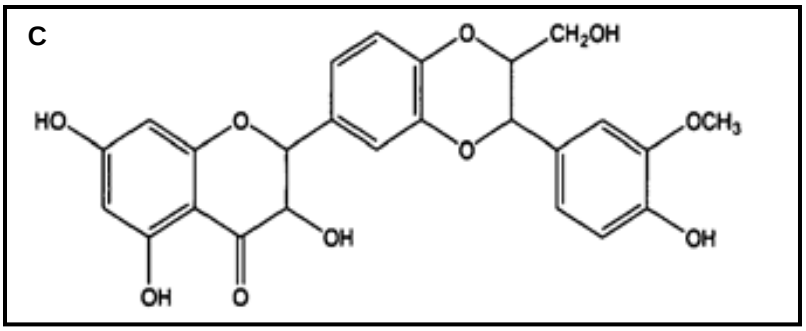
Fisetin

Fisetin (3, 3', 4', 7-tetrahydroxyflavone) is naturally occurring bioactive flavonol found in several fruits and vegetables such as apple, onion, grape, cucumber, strawberry at concentration of range 2-160 µg/g **(Fig.8.)**. The highest concentration of fisetein is found in strawberry (160 µg/g) and apple (26.9 µg/g). Fisetin has been reported as chemopreventive agent in several cancers, although it is also a potent neuroprotective agent. *In vitro* studies have shown the anticancer effects of fisetin in various cancers such as colon, lung, prostate, pancreas and melanoma (Khan et al., 2013).

The overexpression of COX2 and dysregulation of Wnt signaling pathways play important role in colorectal cancer. The treatment of overexpressed COX2 colon cancer cells, HT29 with fisetin at dose of range 30-120 µM, resulted in inhibition of COX2 protein expression with the induction of apoptosis. Treated cells have shown reduced number of colonies assessed through soft agar colony formation assay (Suh et al., 2009). Fisetin is also known to induce apoptosis and decrease clonogenic potential in prostate cancer. The activity of enzyme 5 alpha-reductase is very critical for the development of prostate cancer. Fisetin is a potent inhibitor of the 5 alpha-reductase enzyme activity, thus helpful in prevention of prostate cancer. There are several genes which are involved in G2/M phase cell cycle arrest, apoptosis, stress response and chromosome organization which are modulated fisetin. The modulation of PI3K/Akt and JNK signaling pathways and downregulation of MMP (matrix metalloproteinase) is another significant effects of fisetin implication of their anti-metastatic activity in prostate cancer cells (Adhami et al., 2012).



Structure of Silibinin



Structure of Fisetin

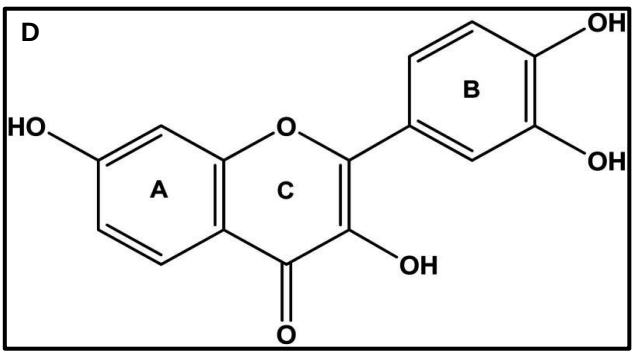


Figure 8. Source of silibinin is milk thistle plant (A), extracted from plant seeds (B). (C) and (D) showing the chemical structure of silibinin and fisetin, respectively.

The invasion and migration ability of cervical cancer cells have been suppressed by fisetin through inhibiton of urokinase plasminogen activator (uPA) activity. It reduced the phosphorylation of p38 MAPK and thus, interrupts the NF-κβ signaling pathway (Chou et al., 2013). Fisetin showed its chemotherapeutic effects where its treatment resulted in decrease expression of anti-apoptotic protein, elevated pro-apoptotic proteins, activation of caspases, disruption of mitochondria membrane potential and release of cytochrome c. The proliferation of cancer cells was inhibited which was accompanied by G2/M arrest (Pal et al., 2013). Melanoma is responsible for more deaths than overall skin cancer. Fisetin is known to promote mesenchymal to epithelial transition in melanoma cells along with their invasion inhibition capacity. Fisetin targets MAPK and NF-κβ signaling pathways which are key regulator pathways involved in melanoma metastasis (Pal et al., 2014).

Overall, the literature has shown that arsenic-induced skin carcinogenesis is a complex process and more understanding of this process has to be deciphered. The most important part is to examine the effect of non-cytotoxic dose of arsenic on skin and explore the molecular alteration associated at specific concentrations so that novel targets for therapeutics could be developed. In our study, we have explored the effect of low concentrations of arsenic on skin cells and their transforming ability of skin cells. Another aspect was to target the transformed skin cell by phytochemicals. In our study, we have demonstrated the effect of phytochemicals, silibinin and fisetin, on the arsenic transformed skin cells.

We further need to explore the effect of arsenic at very low doses on skin cells, so that arsenic mediated molecular mechanisms could be explored and therapeutics could be developed.

Aims and Objectives

3. Aims and objectives

Past few decades, calamities due to arsenic have been increased worldwide especially in India. There are many natural as well as anthropogenic factors attributed for this crisis. This problem cannot be neglected since arsenic found to be elevated in drinking ground water. Since, large population around the globe uses groundwater for their household purposes, it is affecting millions of people using arsenic contaminated groundwater. Also, the grave consequences of arsenic on human health have been seen in individuals exposed to arsenic through drinking water. The permissible limit for arsenic in drinking water for most of the countries is 10 µg/L, as per WHO standards. However, the low dose of arsenic was found in blood of people exposed to high level of arsenic (Snow, Sykora et al. 2005). But, the effect of arsenic at low doses on human is not explored much. Hence, study of effects of arsenic needs to be done at the lower concentrations of arsenic as well as at molecular level. For its clinical relevance, this would help in developing novel strategies for therapeutics and prevention.

3.1. Objectives

Thus, we have proposed following **objectives** for our studies:

1) To examine the effect of arsenic on its transforming ability of HaCaT cells.
2) To study the effects of phytochemicals, silibinin and fisetin, on the transformation of HaCaT cells.
3) To study the effect of arsenic on molecular alterations in HaCaT cells.

Materials and Methods

4. Materials and methods

4.1. Cell culture

HaCaT (Ha = Human adult, Ca = Calcium, T = Temperature) cells, immortalized human keratinocytes which are non-tumorigenic in nature, were purchased from National Centre for Cell Science (NCCS), Pune. These cells were grown in Dulbecco's modifies eagle medium (DMEM) [GIBCO] supplemented with 10% Fetal Bovine serum (FBS) [GIBCO] and 1% penicillin-streptomycin-amphotericin B [Himedia] at 37ºC temperature and 5% CO_2 with 95% humidified ambience maintained by thermoscientific CO_2 incubator. HaCaT cells cultured as adherent monolayer with epithelial morphology. HaCaT cells carries mutation in both allele of p53 gene.

4.1.1. Preparation of media

The incomplete media was prepared by dissolving the powder DMEM in double distilled water to which sodium bicarbonate was added as require (3.7 g/L). Further, media was filtered using 0.22µM filter (Millipore). Finally, the DMEM media was completed by adding 10% FBS and 1% antibiotic to the filtered media.

4.1.2. Revival of cells

The culture media was warmed at 37 ºC in water bath. The ampule was taken from liquid nitrogen storage and thawed at 37 ºC water bath. The ampule (which contains 1ml cell suspension) contents were transferred to the 15ml centrifuge tube which contains 10 volumes of media, which dilutes the cryoprotectant dimethyl sulfoxide (DMSO). Further, the cell suspension was centrifuged at 1500 rpm for 5 minutes and supernatant was discarded. Finally, the pellet was gently resuspended in pre-warmed culture media and transferred in 60 mm petri plates. Then, the plate was incubated at 37ºC and 5% CO_2 in CO_2 incubator. Cells were regularly examined under the phase contrast microscope and were checked for contamination. The media of the plate was changed on every alternative day till the desire confluency was achieved.

4.1.3. Splitting and subculture of cells

When confluency of the cells reached around 70-80%, then splitting of the cells was done. The spent media was aspirated from the plate and the plate was rinsed with 1X Phosphate Buffer Saline (PBS). Then, 1X Trypsin-EDTA (Ethylenediamine tetracetate) [Himedia] was

added on the monolayer of the cells in plate and the plate was incubated at 37 ºC in 5% CO_2 incubator for 5 -10 minutes. The plate was examined under the inverted microscope to check the detachment of cells. After ensuring the detachment, the plate was taken out from the incubator and fresh culture media was added to inactivate the trypsin. The cell suspension was collected from the plate and transferred in the 15 mL falcon tube. The tube was centrifuge at 1000-1500 rpm for 5 minutes at room temperature and supernatant was discarded without disturbing the pellet. The pellet was resuspended in fresh media and transferred to fresh 100 mm culture dish containing pre warmed media. Then, the plate was kept in CO_2 incubator and media was changed on every alternate day till desired confluency was achieved.

4.2. Arsenic, Silibinin and Fisetin Stock preparation

Sodium arsenite, $NaAsO_2$, was purchased from Sigma and used for experiments. The molecular weight of $NaAsO_2$ is 129.91 g. The sodium arsenite was dissolve in double distilled water. The 50 mM stock of sodium arsenite was prepared in water and stored at -20ºC.

Silibinin is one of the main constituent of silymarin which is extacted from plant *Silybum marianum* (milk thistle) (Cheung, Gibbons et al. 2010). The silibinin has molecular weight of 482.44 g which was dissolved in DMSO and stock of 100 mM was prepared and stored in -20ºC.

Fisetin is 3,3′,4′,7-tetrahydroxyflavone with molecular weight of 286.24 g. Fisetin was dissolved in DMSO and stock of 100 mM was prepared, aliquoted and stored at 20ºC. Both silibinin and fisetin were purchased from Sigma Aldrich.

4.3. MTT assay

Reagents Required: MTT **(**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (SRL, India), DMSO (Sigma Aldrich, USA), DMEM (GIBCO) and 1X PBS.

Procedure:

Five thousand cells in volume of 200 µL DMEM media were seeded in each well of 96-well plate and then incubated for 24 hours. Next day, cells were observed under the phase contrast microscope. Different working concentrations of drug were prepared from stock in falcon tube. The spent media from each well of 96-well plate was removed and the treatment media was added in each well with different concentrations. After the treatment

was given for respective time points, the media was removed and 100 µL of MTT (freshly prepared) was added at concentration of 5 mg/mL dissolved in 1X PBS to each well. The 96-well plate was then incubated at 37 ºC in 5% CO_2 incubator for 4 hours. The MTT solution was removed from each well carefully without disturbing the crystals and then 100 µL DMSO was added to each well to dissolve the crystal. Then, the plate was incubated in dark for 10 minutes and the 96-well plate was read in Thermo Scientific Varioskan Flash spectral Scanning Multimode Reader at 570 nm. The data was represented as percent cell viability.

4.4 Trypan blue dye exclusion assay

Reagents Required: 0.4% (w/v) Trypan blue (HIMEDIA), Trypsin-EDTA solution (HIMEDIA) and 1X PBS

Procedure:

Cells were seeded at a density of 2 X 10^4 cells per well of 24 plates in DMEM. The plate was incubated at 37 ºC in 5% CO_2 incubator for 24 hours. Next day, the cells in respective wells were treated with DMSO, vehicle control and different concentration or dose of drugs for specified time periods. After the treatment period, the spent media were collected in 15 mL falcon tubes from respective treatment groups. The cells were trypsinized and collected in respective falcon tubes containing media. Then, the cell suspension was spun at 1500 rpm for 5 minutes. The supernatant was discarded without disturbing the cell pellet. The pellet was washed with 1X PBS and gently resuspended in 1 mL ice cold 1X PBS. One hundred µL of cell suspension taken from respective treatment was aliquoted in micro centrifuge tube and placed on ice. The 10 µL of 0.4% trypan blue dye was mixed gently with cell suspension and incubated at room temperature for 5 minutes (perform in each treatment one by one). Then, after 5 minutes, pipette out 10 µL from the trypan blue dye stained cell suspension and loaded on the both the chambers of the hemocytometer. Cells were counted as live or dead cells using phase contrast microscope at 100X magnification. The unstained cells were counted as live cells while the stained cells were counted as dead cells. Each of the samples counted in duplicate with each treatment counted in triplicate. The cells were scored for live and dead cells and calculated and represented as percent dead cells and total cells. The remaining of 900 µL cell suspension was used for the FACS analysis for cell cycle analysis distribution.

4.5. Clonogenic assay

Reagents: Staining solution- 5 mg/mL crystal violet in 2% ethanol, Fixing solution (12.5% acetic acid and 30% methanol in double distilled water) and 1X PBS.

Procedure:

Five hundred cells in 2 mL DMEM media were seeded in each well of 6-well plate. The cells were the incubated at 37ºC in 5% CO_2 incubator for 24 hours. Next day, the treatment was done in respective wells at different doses and treatment was done in triplicates. The plates were incubated for 7-10 days for the colony formation. Every alternate day, spent media was replaced with fresh media containing drug.

After completion of experiment, the media was aspirated gently without disturbing the colonies and then the plates were washed thrice with 2 mL of 1X PBS. The colonies were then fixed with 2 mL fixative (100% Methanol) in each well for 10 minutes at room temperature. The fixative was gently aspirated out and the wells were then washed with double distilled water thrice. The colonies were stained with 2 mL of the staining solution in each well for approximately 20 minutes. The stain was rinsed off with water without disturbing the colonies till the background got clear and left at room temperature to dry. The colonies were counted under the inverted phase contrast microscope at 100X magnification. Total number of colonies as well as of cells per colony was counted. Colonies were categorized as colonies containing more than 50 cells and less than 50 cells.

4.6. Soft agar colony formation assay

Reagents: Agarose (Low gelling temperature, Sigma), 2X DMEM and Double distilled water.

Procedure:

1% agarose stock made in double distilled water was autoclaved and stored at 4ºC. The stock of 1% agarose was melted in microwave and cooled in 40ºC water bath. The equal volume of 1% agarose and 2X DMEM was mixed (1:1). The 2 ml of this mix was pipetted out and poured in each well of 6-well plate. This layer is known as lower or base layer of agarose (0.5%). Allowed the agarose in each well to set, without disturbing the plate in hood for 30 minutes.

The treated cells were harvested and counted. Ten thousand cells were taken for each well. The volume required for specific number of cells was calculated for all different treatments. The volume of 0.4 mL from 1% agarose solution and 1.1 mL of 2X DMEM containing required number of cells were mixed. The total volume for upper layer was taken 1.5 mL.

This upper layer (0.33%) which contains the cells was mixed and 1.5 mL cells containing 2X DMEM was poured on the base layer in each well. Then, the plate was kept undisturbed for 30 minutes in hood for solidification of the agarose. The 1 mL DMEM media was added on the top of upper layer. Next, the plate was incubated at 37ºC in 5% CO_2 incubator for 20 days. The media of the plate was changed on every alternate day. The colonies formed were observed using inverted phase contrast microscope at 100X magnification. The colonies were counted and plotted as total number of colonies.

4.7. Cell cycle analysis through fluorescence activated cell sorter (FACS)

Reagents: Saponin (Fluka Analyticals, USA), Propidium Iodide (Sigma Aldrich, USA), EDTA (Sigma Aldrich, USA), RNase A (Sigma Aldrich, USA) and 1X PBS.

Procedure:

As discussed in trypan blue assay, the remaining 900 µl cell suspension was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded without disturbing the cell pellet. The cell pellet was resuspended in PI cocktail [PI cocktail consist of 0.2% saponin (w/v), 25 µg/ml PI (w/v), 0.1 mM EDTA and 10 µg/ml RNase (w/v) in 1XPBS] gently in dark conditions. The cells in falcon tubes were incubated overnight at 4ºC in dark conditions. Next day, all the samples were analysed on flow cytometer for 20,000 events per sample and data was acquired using Cell Quest Pro Software in BD FACS Calibur (Becton Dickinson, USA). The data was analyzed using Cell Quest Pro Software and represented in graph as percent cell distribution.

4.8. Reactive oxygen species (ROS) detection using DCFH-DA dye

Reagents: DCFH-DA (Sigma Aldrich), 1X PBS and DMSO.

Procedure:

The fresh 20 mM stock of DCFH-DA was prepared in DMSO in dark condition. The ROS measurement was done by using both spectrophometer as well as through FACS. For spectroscopy, the 15,000 cells were seeded in each well of 96- well plates. The plate was incubated at 37ºC in 5% CO_2 incubator for 24 hours. The spent media was removed from the each well and the DCFH-DA at a concentration of 20 µM was added to each well of the 96 well- plate which incubated at 37 ºC for 30 minutes. After 30 minutes, the media was removed and the well was washed three times with 1X PBS. Then treatment was given to each well respectively and at different time points reading was taken using Thermo Scientific Varioskan Flash spectral Scanning Multimode Reader. For FACS analysis of

ROS generation,, the 3×10^4 cells/well were seeded in 24 well plate in triplicate. The plate was kept in incubator at 37ºC for 24 h. The treatment was given and the DCFH-DA at a concentration of 20 µM was added before 30 minutes of harvesting the cell. Hydrogen peroxide was used as a positive control which was added 15 minutes before harvesting cells. The cells were harvested at a time point of 12 h by the process same as that for trypan blue assay. The pellet was resuspended in 1 ml 1X PBS and ROS production was measured through flow cytometry. The data was acquired using Cell Quest Pro Software in BD FACS Calibur (Becton Dickinson, USA). A minimum number of 10,000 cells were analyzed per sample.

4.9. Cell irradiation using gamma chamber

Reagents: 1X PBS

Procedure:

The arsenic exposed cells were seeded at a density of 2×10^4 cells in 24 well plate and incubated at 37 ºC for 24 h. The cells were grown to near 50-60 % confluency. Before irradiation, the medium was removed from the culture plate and the cells were washed with 1X PBS. A thin layer of 1X PBS covered the cell. The plates were sealed with parafilm before being taken to the irradiator to avoid any contamination. This was followed by irradiation at room temperature in gamma chamber (^{60}C, 240 TBq, Model 4000A) purchased from Bhabha Atomic Research Centre (BARC), Mumbai, India. The dose rate was estimated by Fricke's dosimetry and was found to by 1Gy/30 sec. The cells were irradiated at a dose rate of 5 Gray (Gy) using gamma chamber. Controls cells were identically processed but not irradiated. After irradiation, the plates were wiped with 70% ethanol and taken back in culture hood. 1X PBS was removed form the well and complete media was added to each well. The plates were kept in incubator at 37 ºC for 48 h. After this, trypan blue assay was performed as described earlier and percentage of live and dead cells was plotted on graph.

4.10. Matrix metalloproteinase gelatin zymography

Reagents: Serum-free media (SFM), 2X SDS sample buffer, 0.1% gelatin, Renaturing buffer and zymogram developing buffer.

Procedure:

Cells were grown in 60 mm plates in complete media to 70% confluency followed by treatments with drug for 48 h. After the treatment, the plates were washed with serum free

media (SFM) and SFM was added to the plates for 12 h. The conditioned- medium was collected after 12 h and spun down at 3,000 rpm at 4ºC to remove any cellular debris. Cells were also collected for equilibrating the volume of conditioned media for equal number of live cells in each treatment. Volume of conditioned media from equal equal number of cells was aliquoted from each treatment and mixed with equal volumes of SDS sample buffer (2X) without beta-mercaptoethanol and incubated for 10 minutes at room temperature. 10% SDS-PAGE gels containing 0.1% gelatin (1 mg/ml) in resolving gel were prepared. The samples were prepared without heating and loaded on a gel. The gels were run at a constant voltage of 125 V for 1-1.5 h in 1X Tris-Glycine SDS running buffer. The gels were monitored continuously and stopped when tracking dye bromophenol dye reached at the bottom of the gel. The gels were incubated in renaturing buffer (Appendix F) with gentle agitation for 30 minutes at room temperature in order to remove the SDS from the gel. Last step was done two times to ensure complete removal of SDS. Now, the gels were incubated with 1X zymogram developing buffer (Appendix G) with gentle shaking at 37ºC for 24 h. The gels were stained with coomassie blue R-250 for 1-2 h, which is followed by destaining with destaining solution (Appendix I) with careful monitoring for visible clear bands of protease (MMP-2 and MMP-9) activity appeared against the dark blue background. Gels were scanned using high resolution scanner from Hewlett and Packard, USA.

4.11. RNA isolation from mammalian cells

Reagents: DEPC water, 1X PBS in DEPC, Trizol (from GeNei^{TM}, MERCK), chloroform, isopropanol and 70% ethanol.

Procedure:

The cells were seeded in 100 mm petri dishes and were grown to confluency around 70%. The respective treatment was given for 48 h time period. After 48 h, the spent media was aspirated and the cells in monolayer were washed with ice cold 1X PBS. 1 mL Trizol reagent/100 mm culture dish was added to each plate and plates were kept on ice for 5 minutes. The cells were lysed and removed with cell scrapper and passed the lysate several times through pipette to homogenize the sample. Then, the suspension was transferred to 2 ml centrifuge tubes and incubated for 2-3 minutes. The chloroform (200 µL /mL of Trizol) was added to each tube. The tubes were shaken vigorously for 15 seconds followed by incubation at room temperature (RT) for 2-3 minutes. Then, samples were centrifuged at 12,000 rpm for 15 minutes at 4ºC and upper aqueous phase (contains RNA) was removed carefully without disturbing the interphase and transferred to fresh 1.5 ml tubes. The

isopropanol (500 µL /mL of Trizol) added into the tubes to precipitate the RNA. The tube was incubated overnight in -20ºC and then centrifuged at 12,000 rpm for 10 minutes at 4ºC. The supernatant were discarded and the pellet was washed with 70% ethanol by resuspending it with gentle shaking of tubes 2-3 times followed by centrifugation at 10,000 rpm for 10 minutes at 4ºC. The ethanol was drained off completely and pellet was kept for air dried. The RNA pellet was dissolved in 20 µL DEPC- treated water. The samples were incubated at 56ºC in water bath for 5 minutes for faster dissolution. The quantification of RNA was done using Nanodrop UV spectrophotometric method by measuring the absorbance (A) of the diluted RNA solution. The purity of RNA was checked by taking reading at both at 260 nm and 280 nm. The ratio A_{260}/A_{280} should be 2 or close to 2 for high quality of RNA.

4.12. cDNA synthesis from RNA

Reagents: dNTPs (Thermoscientific), autoclaved Double Distilled water, RNA samples, oligo dT, Reverse Transcriptase enzyme (Thermo Scientific).

Procedure:

The following 25 µL reaction volume was used for 5 µg of total RNA isolated. RNA was denatured alongwith dNTP and buffer at 65ºC for 10 minutes and then immediately kept on ice for 5 minutes. The following is the reaction set up for the cDNA synthesis.

Table 4.1 Standard cDNA synthesis reaction mix

Reagent	Volume (in µL)
RNA (5 µg)	-
5X Reaction buffer	5
dNTPs (10 mM)	2.5
Oligo.dt (0.5 µg/mL)	1
Reverse Transcriptase enzyme	1
DEPC water	to make final volume
Total reaction volume	**25**

4.13. PCR Reaction

The primers were purchased from Polaris, Sigma. Primers provided in lyophilized form were dissolved in autoclaved Milli Q water such that the final concentration of main stock is 1 nmol/µL. Working stocks were prepared by diluting the master stock 100 times (10 pmol/ µL). The following 25 µL reaction was set to carry out 20-25 cycles of regular PCR reaction in thermocycler. The components were added as follows:

Table 4.2. Standard reaction mix for RT-PCR

Reagnets	**Volume (in µL)**
Template (cDNA)	3
Forward Primer (10 pmol/ µL).	1
Reverse Primer (10 pmol/ µL).	1
dNTP mix (10 mM)	1
10X PCR buffer containing Mg^{2+}	2.5
Taq DNA polymerase	0.5
Autoclaved Double distilled water	16
Total Reaction Volume	**25**

The PCR cycles

Step 1- Initial denaturation at 95ºC for 3 minutes

Step 2 - Denaturation at 94ºC for 30 seconds

Step 3- Annealing at 55ºC for 30 seconds*****

Step 4- Extension at 72ºC for 1.30 minutes

} 25-30 cycles

Step 5- Final elongation at 72ºC for 5-7 minutes

Step 6- Store at 4ºC

*****Annealing temperature was adjusted depending upon the primers.

PCR products were analyzed using 1-1.5% agarose gel electrophoresis prestained with ethidium bromide and visualized under the GELDOC system (Applied Biosystems, USA).

List of primers used in experiment:

Gene	Forward Primer	Reverse Primer
BCL2A1	5'-GCTGGCTCAGGACTATCTGC-3'	5'-ATCCACATCCGGGGCAATTT-3'
TNC	5'-GAGATGCCAAGACTCGCTACA-3'	5'-GTTGACACGGTGACAGTTCCT-3'
RAB31	5'-CGAGCACATGATGGCGATACG-3'	5'-GTCCTTCAGCAGTGCACAGGA-3'
IGFL1	5'-GGCTGCATCGTAGCTGTCTT-3'	5'-CAAAGCCTGTCATCCGAGGT-3'
RGL4	5'-AACTGCATCACCACCTCCTG-3'	5'-TTTCCTGCAGAACTCGGACC-3'
ERbB2	5'-GCAGGATATCCAGGAGGTGC-3'	5'-GCCAGCTGGTTGTTCTTGTG-3'

4.14. Statistical Analysis

All the data were analyzed for statistical analysis using GraphPad Prism software. Student's *t*-test was used to determine the significance difference between different groups and $p<0.05$ value was considered significant. One way ANOVA test was used for multiple comparisons.

Results

5. Results

5.1. Arsenic induced proliferation in HaCaT cells at non-cytotoxic dose

A range of arsenic concentrations, from low to high dose, were screened for its effect on the HaCaT cells. HaCaT cells were used for the experiment because it is well studied and established model for studying effect of arsenic on skin keratinocytes. HaCaT cells are transformed cell lines, having characteristics of normal skin cells, with no malignant properties. Effect of different concentrations of arsenic on HaCaT cells were screened through MTT assay. Under regular growth conditions, arsenic treatment (0.05, 0.1, 0.5, 1 and 3 µM) resulted in different responses at different concentrations in HaCaT cells. Low dose of arsenic, 0.05, 0.1 and 0.5 µM, resulted in increased percent cell viability while higher dose of arsenic, 3 µM, resulted in decreased in percent cell viability, at 24, 48 and 96 hours. Dose of arsenic of 0.1 µM resulted in 6%, 8% and 29% ($p<0.05$) increased in percent cell viability as compared to control at 24, 48 and 96 hours, respectively while arsenic dose of 0.5 µM resulted in 3%, 3% and 32% ($p<0.05$) increased in percent cell viability as compared to control at 24, 48 and 96 hours, **(Fig.9 A, B, C)**. At dose of 3 µM, arsenic caused decrease in percent cell viability by 4%, 13% and 13% as compared to control at 24, 48 and 96 hours, respectively. These results suggest that arsenic has biphasic response in HaCaT cells and attributes to varying outcomes at different concentrations. At low dose, there was increased in cell proliferation in HaCaT cells without toxicity, while at high dose it is toxic to the cells.

5.2. Increased proliferation upon arsenic treatment in HaCaT cells grown in serum starved condition

Effect of various doses of arsenic (0.05, 0.1, 0.5, 1 and 3 µM) on HaCaT cells grown in starved conditions (1% FBS) were assessed using MTT assay. Since, arsenic induced proliferation in HaCaT cells in normal growth media (10% FBS), treatment of arsenic to HaCaT cells grown in starved condition (1% FBS) was assessed and found that it also induced proliferation in starved conditions as compared to control. We observed that dose of arsenic, 0.5 µM, resulted in 8% ($p<0.05$) and 15% ($p<0.05$) increase in percent cell viability as compared to control (1% FBS) at 48 and 96 hours, respectively **(Fig.10 A, B, C)**. In starved condition, percent of cell viability of control is reduced to 26% and 55%, as compare to control (10% FBS) at 48 and 96 hours, respectively **(Fig.10 A, B, C)**. There were no significant effects of arsenic on HaCaT cells with other doses of arsenic. Further,

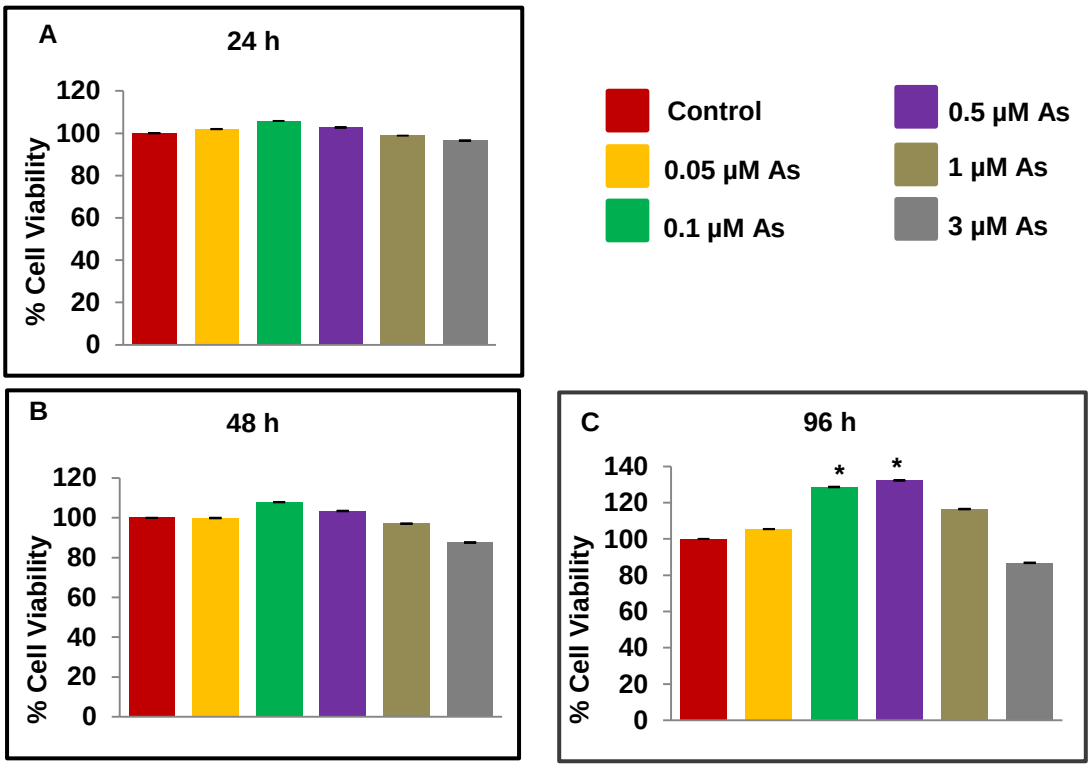


Figure 9. Effect of arsenic on HaCaT cells in MTT assay at different concentrations. HaCaT cells were grown in 96-well plates for 24 h and treated with different concentrations of arsenic (0.05, 0.1, 0.5. 1 and 3 µM). Effect of arsenic on HaCaT cells were observed at 24 h (A), 48 h (B) and 96 h (C). MTT assay was performed to evaluate the cell viability as described in materials and methods. Data are shown as mean ± SE of three independent wells and is representation of 4 independent experiments. P value: $p<0.05$ (*) . As: Arsenic.

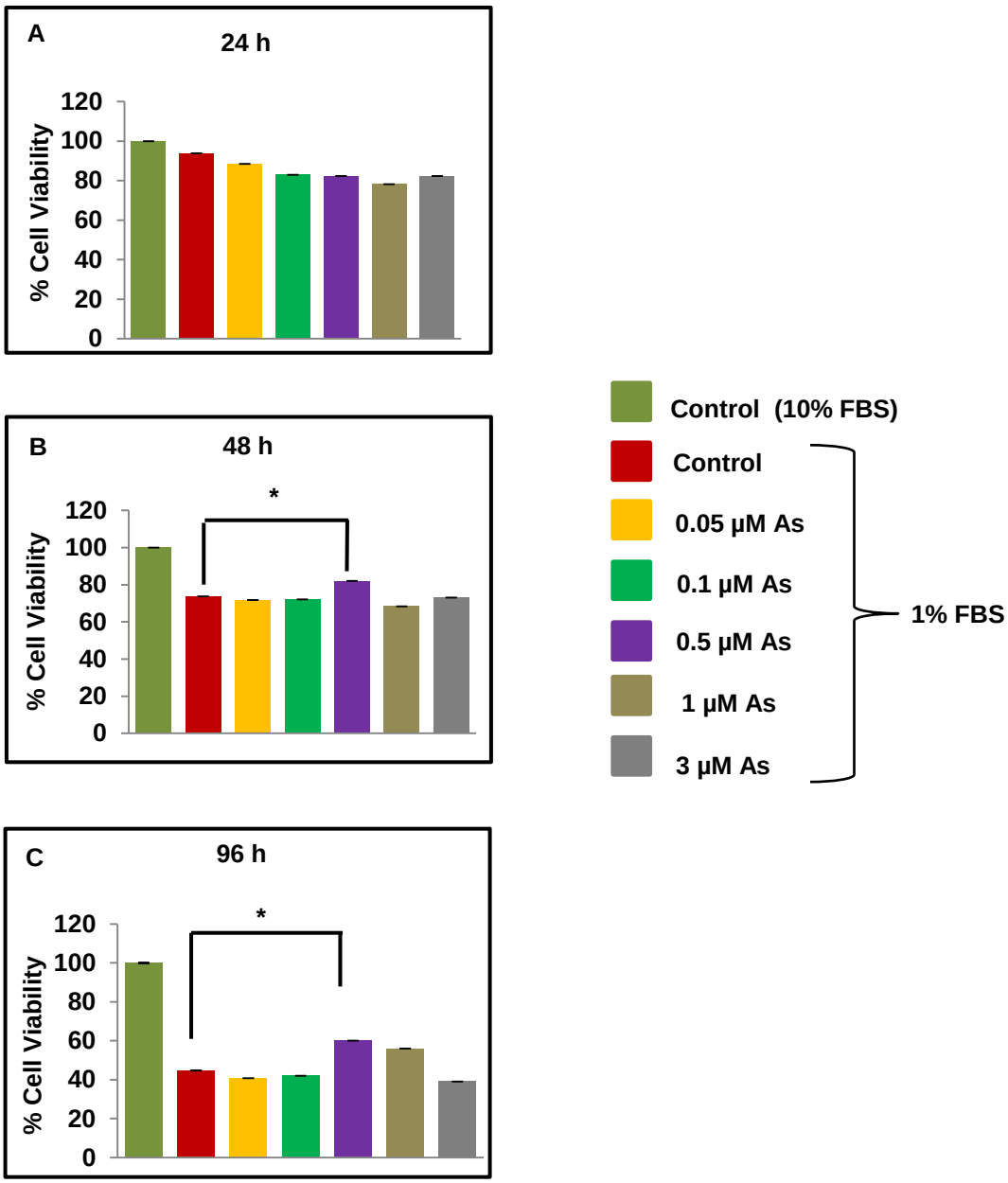


Figure 10. Effect of arsenic on HaCaT cell proliferation in starved condition. HaCaT cells were grown in 1% FBS in 96-well plates for 24 h and treated with different concentrations of arsenic (0.05, 0.1, 0.5. 1 and 3 µM) in 1% FBS. Effect of arsenic on HaCaT cells in starved conditions were observed at 24 h (A), 48 h (B) and 96 h (C). MTT assay was performed to evaluate the cell viability as described in materials and methods. Data are shown as mean ± SE of three independent wells and is representation of 4 independent experiments. P value: $p<0.05$ (*) . As: Arsenic.

together these results showed that the arsenic induced proliferation in HaCaT cells early at 48 hours in both regular as well as starved conditions.

5.3. Effect of arsenic on the clonogenic potential of HaCaT cells

Clonogenic assay or colony formation assay is an *in vitro* method to detect a colony formed from single cell. A colony containing 50 or more cells is usually counted as one or single colony. Clonogenic potential determines the potential of single cell that undergo unlimited cell division, and form a colony. Therefore, we examined the effect of arsenic on clonogenic potential of HaCaT cells. HaCaT cells were treated with arsenic at dose, 0.05, 0.1 and 0.5 µM, for 7 days alongwith control. We observed 15% reduction in total number of colonies including less than fifty cells per colony at 0.5 µM arsenic as compared to control while 43% ($p<0.01$) reduction in colonies (>50 cells) in 0.5 µM as compared to control **(Fig.11)**. There were no significant changes in number of colonies observed at 0.05 and 0.1 µM doses of aresnic as compared to control **(Fig.11)**. Dose of arsenic, 1 and 3 µM, are toxic to the cells with few colonies formed in the plate (data not shown). Overall, arsenic did not affect the clonogenic potential of HaCaT cells at lower doses, however, at dose 0.5 µM arsenic, inhibited the clonogenic potential of HaCaT cells only in colonies (>50 cells) but no reduction was observed in total number of colonies. Hence, result suggests that lower doses of were non-cytotoxic to the HaCaT cells and 0.5 µM arsenic inhibited the clonogenic potential of these cells.

5.4. Arsenic mediates anchorage independent growth, and thus transformed HaCaT cells

Transformation is the process of certain changes, for example genetic alterations etc, in the cells favoring them to behave differently from normal cells. Soft agar colony formation assay is a well-established assay for selection of transformants cells from normal cell. This assay provides the three dimensional structure that is made from different percentage of agar, so that cells suspended in the agar. Since, there is no plane for growing normal cells, only transformant/malignant cells will able to grow there and form a colony.

So, next we explored that whether arsenic at low dose transformed HaCaT cells, for that, we have performed soft agar assay for selection of transfomants in HaCaT cells exposed to arsenic for one month at dose 0.1 and 0.5 µM. After 20 days, we found change in number of colonies as percent of control at arsenic dose, 0.1 and 0.5 µM ($p<0.05$), were 304 and 324, respectively when compared to control **(Fig.12)**. The size of colony at 0.5 µM was found to

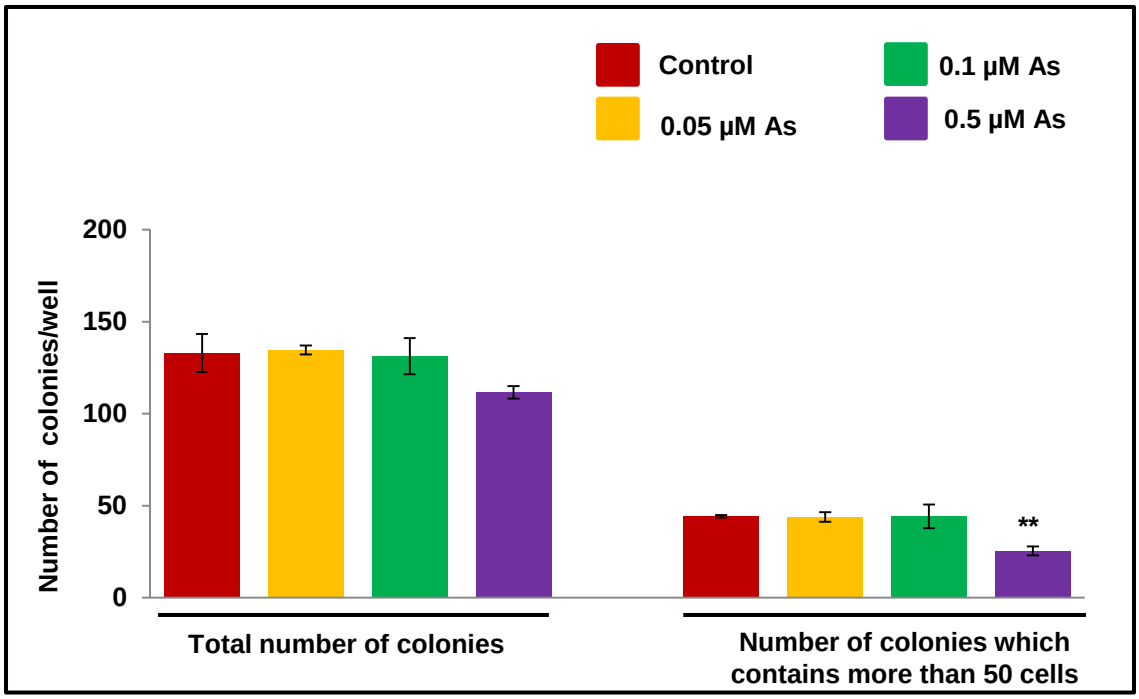
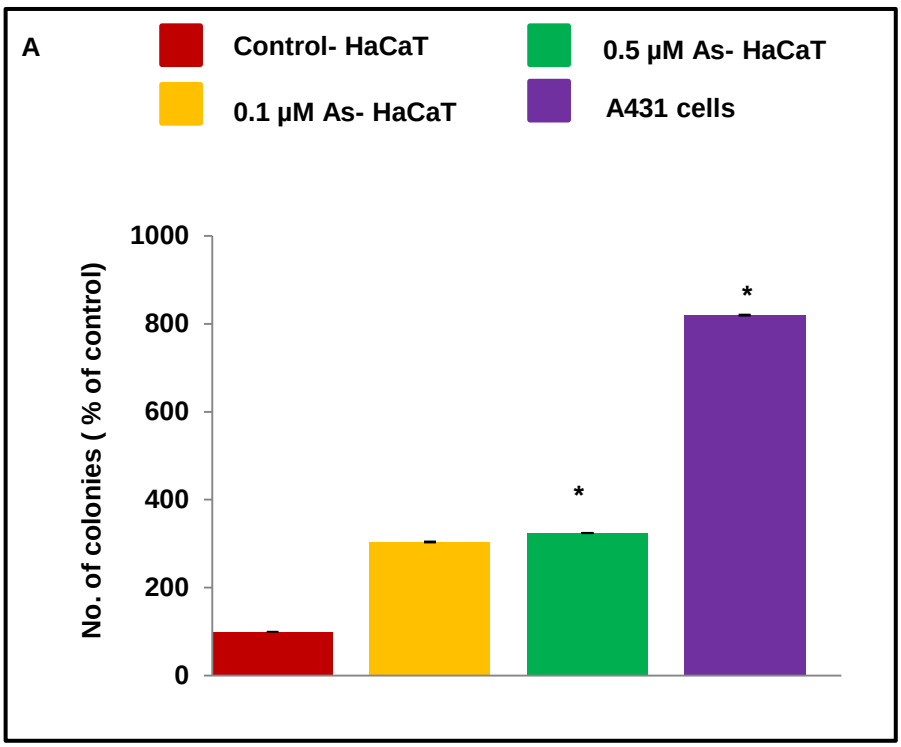


Figure 11. Effect of arsenic on the clonogenic potential of HaCaT cells. HaCaT cells were seeded at 300 cells/well of 6-well culture plate and grown till 24 h. Cells were treated with different concentrations of arsenic (0.05, 0.1 and 0.5 µM) for 7 days. Every 3rd day, fresh treatment media was added to respective wells. At the end of the 7th day of the treatment, cells were stained with crystal violet dye. Quantitative data of the total number of colonies and colonies containing more than 50 cells were represented as a graph. The experiments were done in triplicates, and data are shown as mean ± SE of three independent wells and is representation of 4 independent experiments. P value; $p<0.01$(**) As: Arsenic.



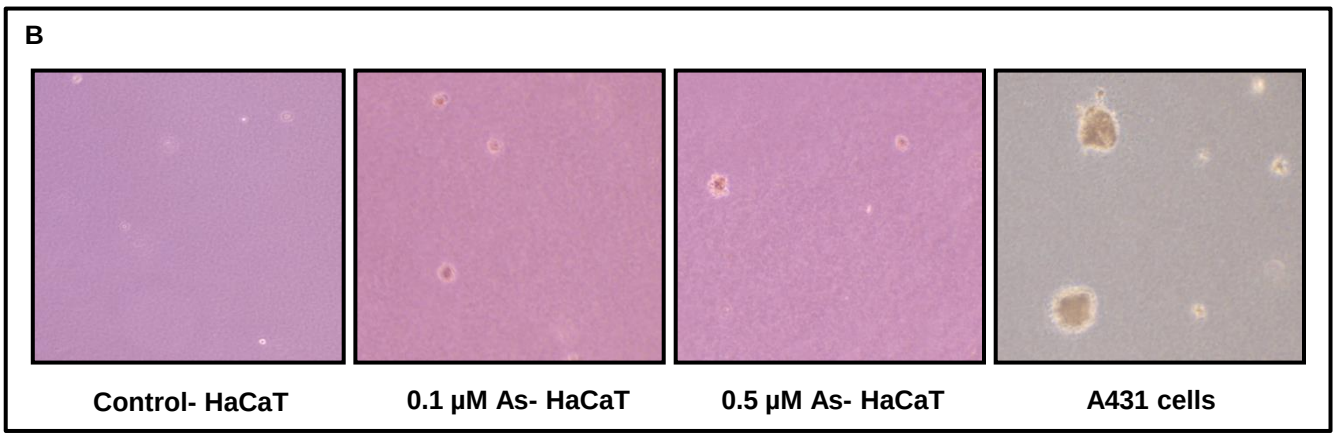


Figure 12. Effect of arsenic on transformation of HaCaT cells. HaCaT cells exposed to arsenic for 1 month have been used for the soft agar colony formation assay. Cells were exposed to arsenic at concentrations of 0.1 and 0.5 µM. Ten thousands (10^4 cells/well) cells were mixed with agarose (0.3%) to make upper layer and pour onto the lower layer of agarose (0.5%) in 6-well plates. A431 cells were used as a positive control. Plates were kept for 20 days, with replacing the spent media on every 3rd day. After 20 days, colonies were counted in phase contrast microscope at different fields. Quantitative data of the total number of colonies were represented as a graph (A). (B) Representative pictures of the colonies in different treatments. Data are shown as mean ± SE of three independent wells and is representation of 2 independent experiments . P value: $p<0.05$ (*). As: Arsenic

be bigger as compared to 0.1 µM. A431 cells were used as a positive control and change in percent of control was found to be 820 when compared to control **(Fig.12)**. This result suggests that chronic exposure of arsenic at non-cytotoxic is able to transform the HaCaT cells. Treatment of HaCaT cells with arsenic at non-cytotoxic dose for 48 hours was also able to transform HaCaT cells, although number of transformants were very low (data not shown).

5.5 Phytochemicals, silibinin and fisetin, modulates acute effect of arsenic on HaCaT cells

Till now, we observed that arsenic transformed the HaCaT cells, hence increased their proliferation rate. We used phytochemicals, silibinin and fisetin, for targeting arsenic-induced transformed cells and their proliferation. Effect of arsenic at dose, 0.1 and 0.5 µM, and phytochemicals, silibinin and fisetin, alone and in combinations on HaCaT cells was assessed using MTT assay at 48 hours of treatments. At 48 hours, arsenic dose 0.1 and 0.5 µM resulted in 10% ($p<0.01$) and 3% ($p<0.05$), respectively, increase in cell viability as compared to control **(Fig.13)**. Silibinin (100 µM) and fisetin (25 µM), alone shown 47% and 41% reduction in percent cell viability as compared to DMSO treated vehicle control at 48 hours **(Fig.13)**. The proliferative effects of arsenic at dose 0.1 and 0.5 µM were inhibited when co-treated with phytochemicals, silibinin and fisetin. Inhibition of proliferation of HaCaT cells at arsenic dose of 0.1 µM observed, and percent cell viability was decreased 57% ($p<0.001$) and 59% ($p<0.001$), for silibinin and fisetin respectively, as compared HaCaT cells grown in arsenic dose 0.1 µM alone **(Fig.13)**. Similarly, the strong inhibition was observed when dose of arsenic 0.5 µM, co-treated with phytochemicals, silibinin and fisetin. We observed 48% ($p<0.001$) and 46% ($p<0.001$) inhibition in HaCaT cells grown at arsenic dose of 0.5 µM, with silibinin and fisetin, respectively, as compared HaCaT cells grown in arsenic dose 0.5 µM alone **(Fig.13)**.

Further, the inhibitory effects of phytochemicals on arsenic-induced proliferation were assessed quantitatively through trypan blue assay. The 0.1 and 0.5 µM doses of arsenic resulted in 51% ($p<0.05$) and 31% ($p<0.05$) increases in cell number at the 48 hour treatment, respectively **(Fig.14 A)**. Silibinin (100 µM) and fisetin (25 µM) alone, caused 39% and 61%, decrease in cell number as compared to DMSO vehicle control, respectively. Strong inhibition of phytochemicals was found on arsenic treated cells. Effect of silibinin and fisetin on total cell number inhibition was found to be 67% ($p<0.05$) and 81% ($p<0.01$), respectively, as compared to HaCaT cells grown in arsenic dose 0.1 µM alone **(Fig.14 A)**.

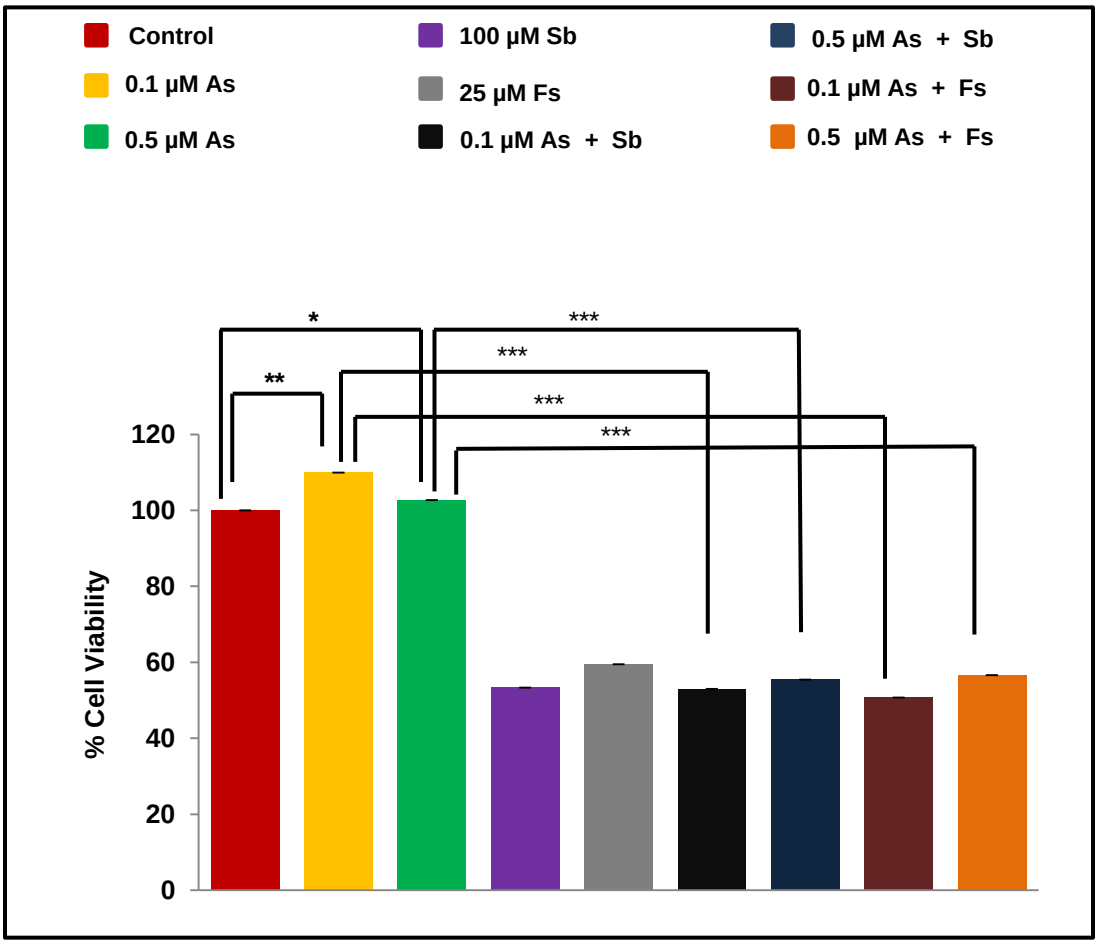


Figure 13. Silibinin and Fisetin modulate arsenic-induced proliferation of HaCaT cells. HaCaT cells were grown in 96-well plates for 24 h and then treated with different concentrations of arsenic (0.1 and 0.5 µM) alone or in combination with phytochemicals, silibinin or fisetin, for 48 h. MTT assay was performed to evaluate the cell viability as described in materials and methods. Data are shown as mean ± SE of three independent wells and is representation of 3 independent experiments. P value: $p<0.05$ (*) , $p<0.01$(**) and $p<0.001$(***). As: Arsenic, Sb: Silibinin, Fs: Fisetin

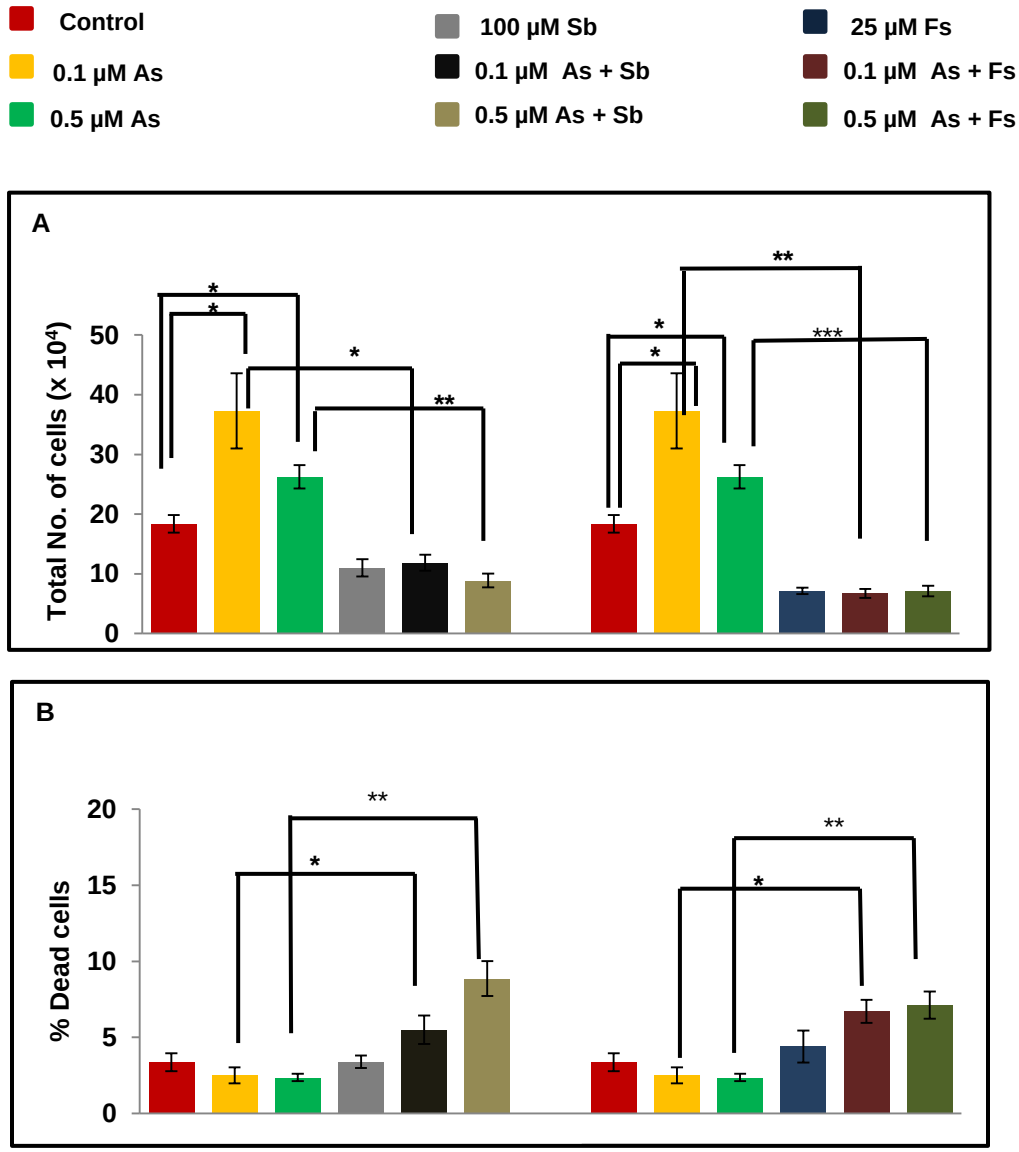


Figure 14. Effect of phytochemicals, Silibinin and Fisetin, on arsenic-induced proliferation of HaCaT cells. HaCaT cells were grown in 24-well plates for 24 h. Cells were treated with different concentrations of arsenic (0.1 and 0.5 µM) alone and in combination with phytochemicals, silibinin and fisetin, for 48 h. Trypan blue assay was performed to evaluate total number of cells and percent of dead cells as described in materials and methods.. (A) Quantitative data are shown for the total number of cells in arsenic alone and in combination with phytochemicals after 48 h. (B) Quantitative data are shown for percent dead cells in arsenic alone and in combination with phytochemicals after 48 h. Data are shown as mean ± SE of three independent wells and is representation of 3 independent experiments. P value: $p<0.05$ (*) , $p<0.01$(**) and $p<0.001$(***). As: Arsenic, Sb: Silibinin, Fs: Fisetin

Similarly, at arsenic dose 0.5 µM, 65% ($p<0.01$) and 73% ($p<0.001$) cell number decreases were shown by silibinin and fisetin, respectively, as compared to HaCaT cells grown in arsenic dose 0.5 µM alone **(Fig.14 A)**. In terms of cell death, there were no significant changes in percent cell death in control, 0.1 µM and 0.5 µM, as expected, since these were non-cytotoxic doses as proved earlier. However, we found 5% ($p<0.05$) and 7% ($p<0.05$) cell death with silibinin and fisetin, respectively, with arsenic dose of 0.1 µM as compared to HaCaT cells grown in arsenic dose of 0.1 µM alone **(Fig.14 B)**. Similarly, in combination with arsenic dose 0.5 µM, 9% ($p<0.01$) and 7% ($p<0.01$) cell death were shown by silibinin and fisetin, respectively, as compared to HaCaT cells grown in arsenic dose of 0.5 µM **(Fig.14 B)**. Therefore, these results suggest that phytochemicals, silibinin and fisetin, strongly inhibited the proliferation of arsenic transformed HaCaT cells.

5.6. Morphological changes in HaCaT cells on treatment with phytochemicals

Effect of arsenic and phytochemicals on the morphology of HaCaT cells was observed. HaCaT cells are epithelial in nature, normally grown as cuboidal shape in packed form. We observed no morphological changes in HaCaT cells when treated with 0.1 and 0.5 µM doses of arsenic at 24 hours, as compared to control (**Fig.15 B and C)**. Treatment of silibinin (100 µM) changed the shape of the HaCaT cells form normal cuboidal shape to round or spindle shape with less packed, with combination of arsenic dose of 0.1 and 0.5 µM at 24 hours (**Fig.15 F and H)**. Similarly, treatment of fisetin (25 µM) changed the shape of the HaCaT cells form normal cuboidal shape to round or spindle shape with less packed, at arsenic dose of 0.1 and 0.5 µM at 24 hours (**Fig.15 G and I)**. These morphological changes on treatment with phytochemicals suggest cell death caused by these phytochemicals, alongwith decrease in the overall number of adherent cells, and thus inhibition of cell proliferation.

5.7. Induction of cell cycle arrest by silibinin and fisetin in arsenic transformed HaCaT cells

As we observed growth inhibitory effect of phytochemicals on the arsenic transformed HaCaT cells, we then analysed for the effect of arrest of cell cycle progression using flow cytometer technique. HaCaT cells were treated with arsenic at doses 0.1 and 0.5 µM alone and in combination with phytochemicals silibinin (100 µM) and fisetin (25 µM) for 48 hours. As we observed strong inhibition of arsenic-transformed HaCaT cells through phytochemicals, there was increased G1 and G2/M arrest observed at doses of arsenic, 0.1

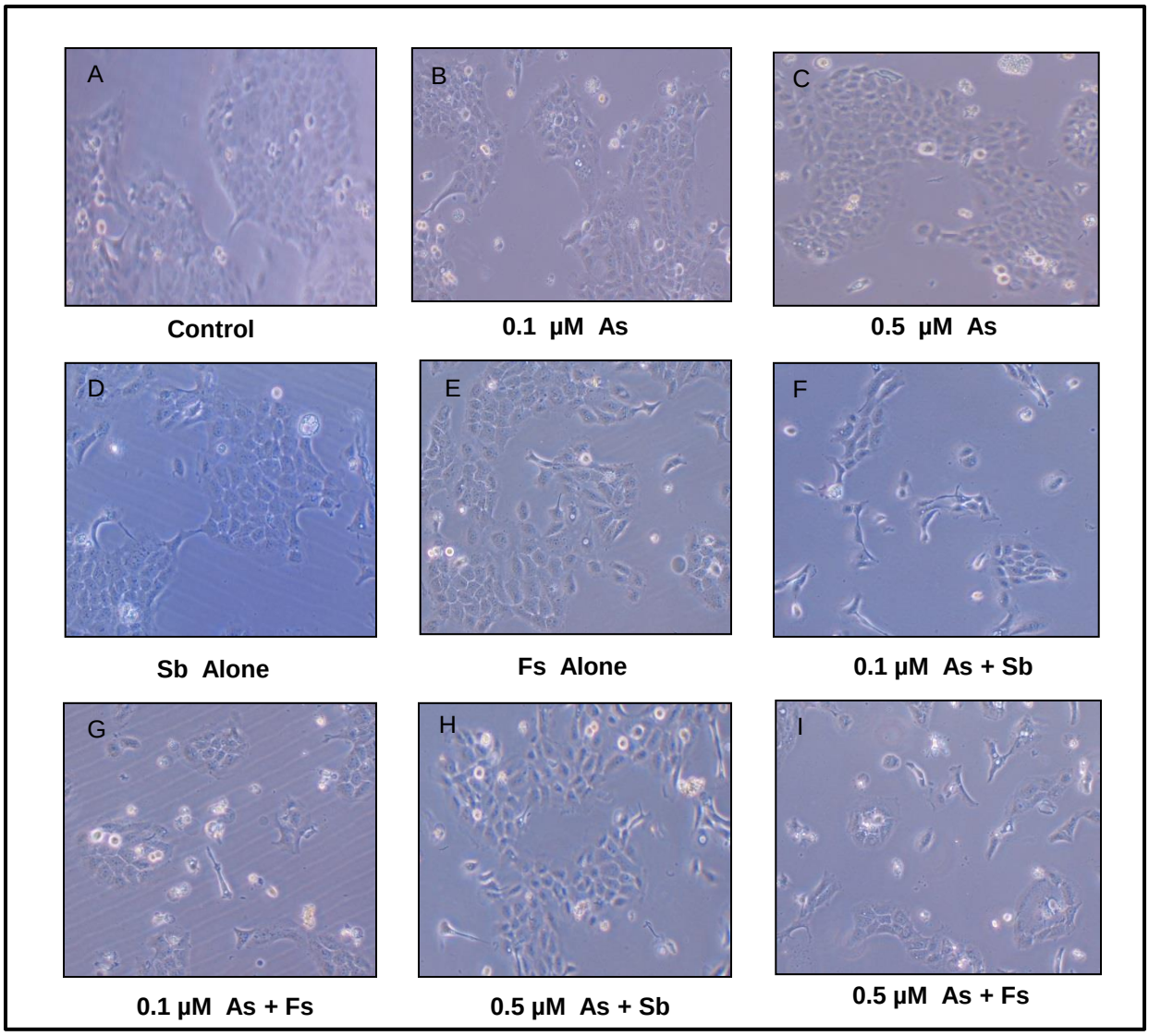


Figure 15. Effect of phytochemicals, Silibinin and Fisetin, on cell morphology in the presence of arsenic. Representation of morphology of HaCaT cells with 0.1 µM As and with phytochemicals and 0.5 µM As and with phytochemicals after 24 h of treatment at 100X magnification.

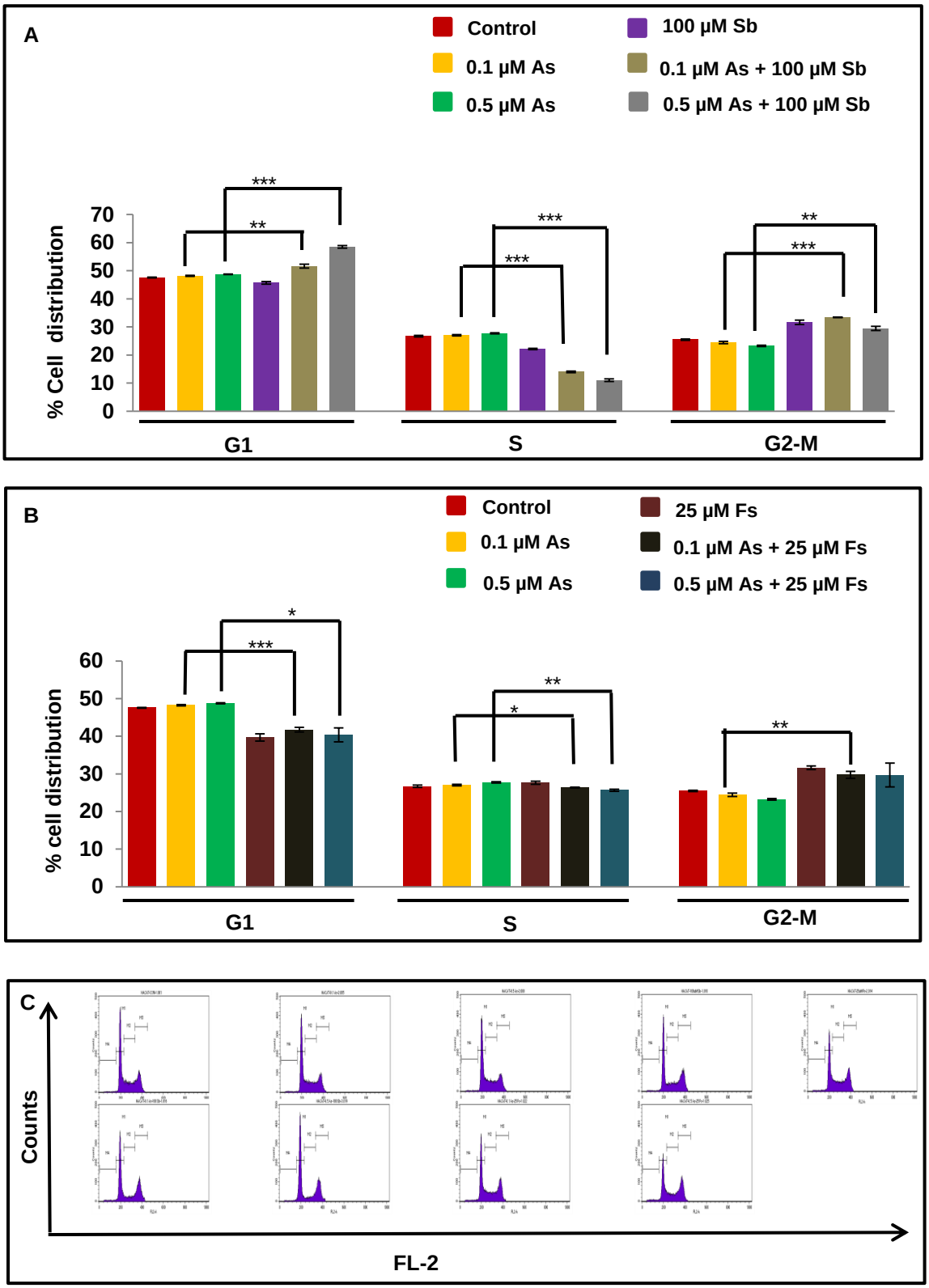


Figure 16. Phytochemicals, Silibinin and Fisetin, induces G1 and G2/M cell cycle arrest in arsenic transformed HaCaT cells. (A) Quantitative data for HaCaT cells treated with arsenic and silibinin alone and in combination after 48 h. (B) Quantitative data for HaCaT cells treated with arsenic and fisetin alone and in combination after 48 h. (C) Cell cycle analysis was done using Propidium iodide by flow cytometry. Data are shown as mean ± SE of three independent wells and is representation of 1 independent experiments. P value: $p<0.05$ (*) , $p<0.01$(**) and $p<0.001$(***). As: Arsenic, Sb: Silibinin, Fs: Fisetin

and 0.5 µM, when treated of silibinin, as compared to respective treatment at 48 hours. (**Fig.16 A)**. There was significant decrease in S phase population at both doses of arsenic treated with phytochemicals. In case of fisetin, we observed G2/M arrest at both doses of arsenic, 0.1 and 0.5 µM, as compared to respective treatment at 48 hours (**Fig.16 B)**. There was a significant decrease in G1 phase population in both doses of arsenic treated with phytochemicals. Together, these observations suggest that phytochemicals, silibinin and fisetin, caused growth inhibition in transformed HaCaT cells involving modulation of cell cycle progression.

5.8. Growth kinetics of chronically exposed HaCaT cells

Next we examined the chronic effect of arsenic on HaCaT cells. For that, we continuously exposed the HaCaT cells to arsenic at dose of 0.1 and 0.5 µM for one month. After this, cells were freezed and later on used for the experiments. Next, growth kinetics studies were done on the exposed transformed cells and graph was plotted. We observed 47% ($p<0.01$) and 36% ($p<0.001$) increase in total number of cells in 0.1 µM exposed HaCaT cells after 72 and 96 hours, respectively, as compared to control (**Fig.17)**. While no significant changes were seen in HaCaT cell exposed to 0.5 µM as compared to control. So, we found that the chronic exposure of 0.1 µM arsenic dose caused more proliferation while chronic exposure at 0.5 µM arsenic dose, might have caused the stress and damage in the HaCaT cells so that no proliferation was observed as seen in acute exposure. Also, we found that there was no reversal in arsenic effects when exposed HaCaT cells were grown in arsenic-free media. In conclusion, arsenic induced transformation effect on HaCaT cells is irreversible.

5.9. Phytochemicals, silibinin and fisetin, modulates chronic effect of arsenic on HaCaT cells

After chronically exposing the HaCaT cells for one month, transformed cells were targeted using phytochemicals, silibinin and fisetin and effects were assessed through trypan blue assay. Arsenic exposed HaCaT cells at 0.1 µM resulted in 17% increase in cell number in the 48 hour as compared to control while there was no significant proliferation was observed in arsenic exposed HaCaT cells at 0.5 µM **(Fig.18 A)**. Silibinin (100 µM) or fisetin (25 µM) alone, caused 56% and 68%, decrease in cell number as compared to DMSO vehicle control, respectively. Effect of silibinin and fisetin on HaCaT cells, exposed at arsenic dose 0.1 µM, was found to be 63% ($p<0.01$) and 81% ($p<0.01$), respectively,

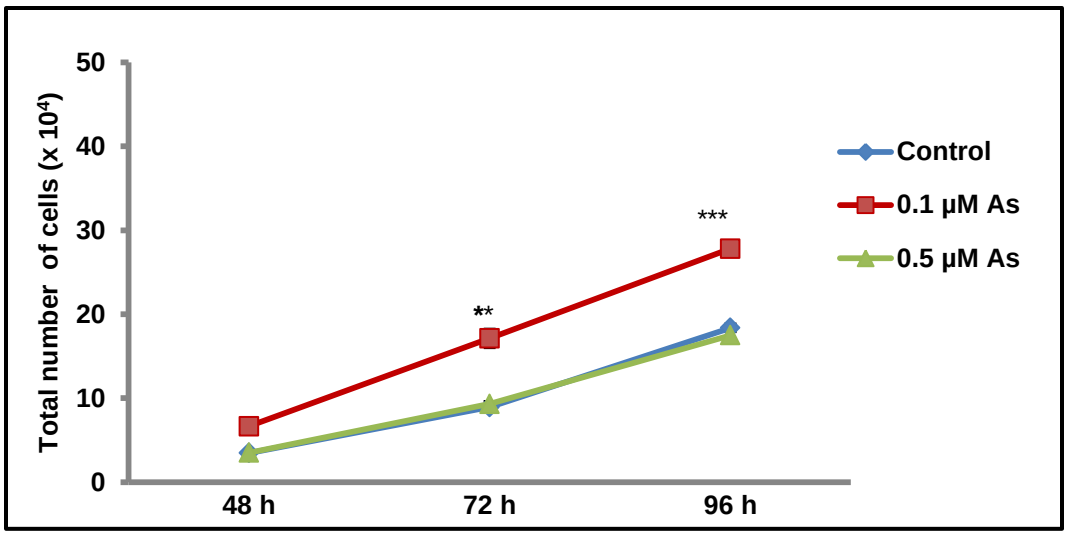


Figure 17. Growth kinetics curve of transformed HaCaT cells exposed to arsenic for one month. HaCaT cells exposed chronically to arsenic with concentration of 0.1 and 0.5 µM for one month. Freezed transformed vials were taken out and revived. Experiments were performed using these cells. 2×10^4 cells were seeded in 6-well culture plate for each condition with control. Total number of different cells were counted at 48 h, 72 h and 96 h and represented as a graph. Data are shown as mean ± SE of three independent wells and is representation of 2 independent experiments. P value: $p<0.05$ (*) , $p<0.01$(**) and $p<0.001$(***). As: Arsenic.

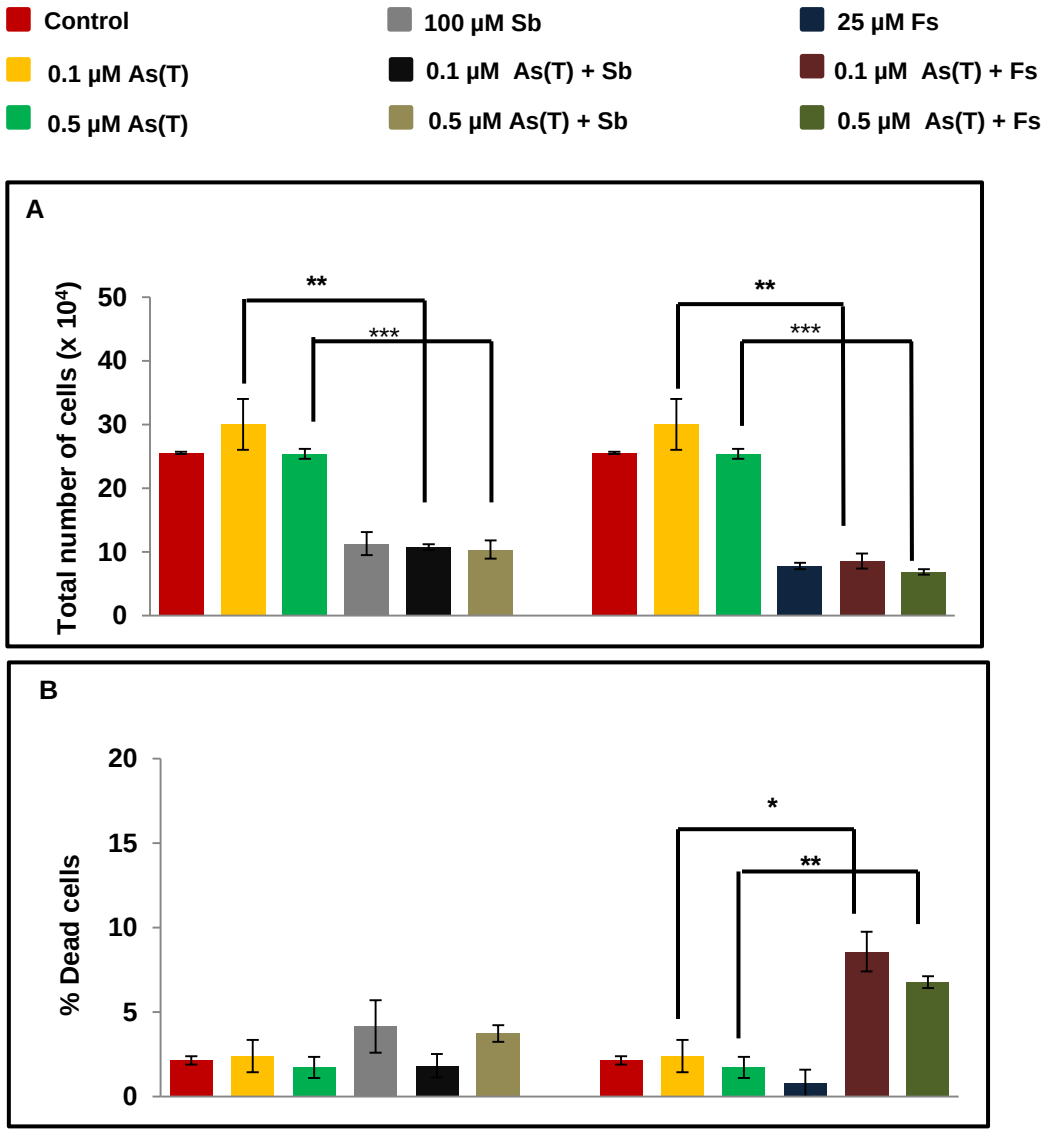


Figure 18. Effect of phytochemicals, Silibinin and Fisetin, on HaCaT cells exposed to arsenic for one month. Transformed HaCaT cells, exposed to 0.1 and 0.5 µM concentration of arsenic, grown in 24-well plates for 24 h. Cells were treated with phytochemicals, silibinin and fisetin, for 48 h. Trypan blue assay was performed to evaluate total number of cells and percent of dead cells as described in materials and methods.. (A) Quantitative data are shown for the total number of cells in arsenic- transformed cells alone and in combination with phytochemicals after 48 h. (B) Quantitative data are shown for percent dead cells in arsenic -transformed cells alone and in combination with phytochemicals after 48 h. Data are shown as mean ± SE of three independent wells and is representation of 2 independent experiments. P value: $p<0.05$ (*) , $p<0.01$(**) and $p<0.001$(***). As(T): Arsenic Transformed HaCaT cells exposed to arsenic for one month. Sb: Silibinin, Fs: Fisetin

decreased in total cell number as compared to untreated exposed HaCaT cells alone **(Fig.18 A)**. Similarly, arsenic exposed HaCaT cells at dose 0.5 µM, shown 60% ($p<0.01$) and 72% ($p<0.001$) decrease in cell number through silibinin and fisetin, respectively, as compared to untreated exposed HaCaT cells alone **(Fig.18 A)**. We found 8% ($p<0.05$) and 7% ($p<0.01$) cell death at arsenic doses 0.1 µM and 0.5 µM, respectively, when treated with fisetin. **(Fig.18 B)**. Overall, these results suggest that chronic effects of arsenic on HaCaT cells could be mitigated using phytochemicals.

5.10. Combinatorial approach using phytochemicals and gamma radiation to alleviate chronic effect of arsenic on HaCaT cells

Next, we used combinatorial approach to target the transformed HaCaT cells. Gamma radiation, with dose of 5 gray (5 Gy), was used in combination with phytochemicals to target transformed cells. HaCaT cells exposed to arsenic for one month at dose 0.1 and 0.5 µM were used for the experiments and the effects were assessed using trypan blue assay. Arsenic exposed HaCaT cells at 0.1 µM resulted in 13% increases in cell number in the 48 hour as compared to control while there was no significant proliferation was observed in arsenic exposed HaCaT cells at 0.5 µM **(Fig.19 A)**. Effect of silibinin (100 µM) on 0.1 µM exposed HaCaT resulted in 69% decrease in total cell number while the combination effect of silibinin and gamma radiation resulted in 79% reduction in total cell number as compared to exposed HaCaT cells at 0.1 µM arsenic alone. Similarly, treatment of silibinin to 0.5 µM exposed HaCaT cells resulted in 59% inhibition in cell number while in combination with radiation resulted in 77% reduction in total number of cells as compared to exposed HaCaT cells at 0.5 µM alone **(Fig.19 A)**. Alone effect of gamma radiation on exposed HaCaT cells was found to be 10% in both 0.1 and 0.5 µM exposed cells, that is comparable to phytochemicals treatment. In terms of cell death, combination of silibinin and radiation resulted in 4% ($p<0.05$) cell death in 0.1 µM exposed HaCaT cells, as compared to 1% cell death in silibinin alone at 0.1 µM exposed HaCaT cells **(Fig.19 B)**. Similarly, combination of silibinin and radiation resulted in 9% cell death in 0.5 µM exposed HaCaT cells, as compared to 3% cell death in silibinin alone at 0.5 µM exposed HaCaT cells **(Fig.19 B)**.

Further, we assessed the combinatorial approach to target the transformed HaCaT cells with phytochemical fisetin. Arsenic exposed HaCaT cells at 0.1 µM resulted in 13% increases in cell number in the 48 hour as compared to control while there was no significant proliferation observed in arsenic exposed HaCaT cells at 0.5 µM **(Fig.20 A)**. Effect of fisetin (25 µM) on 0.1 µM exposed HaCaT resulted in 72% decrease in total cell number

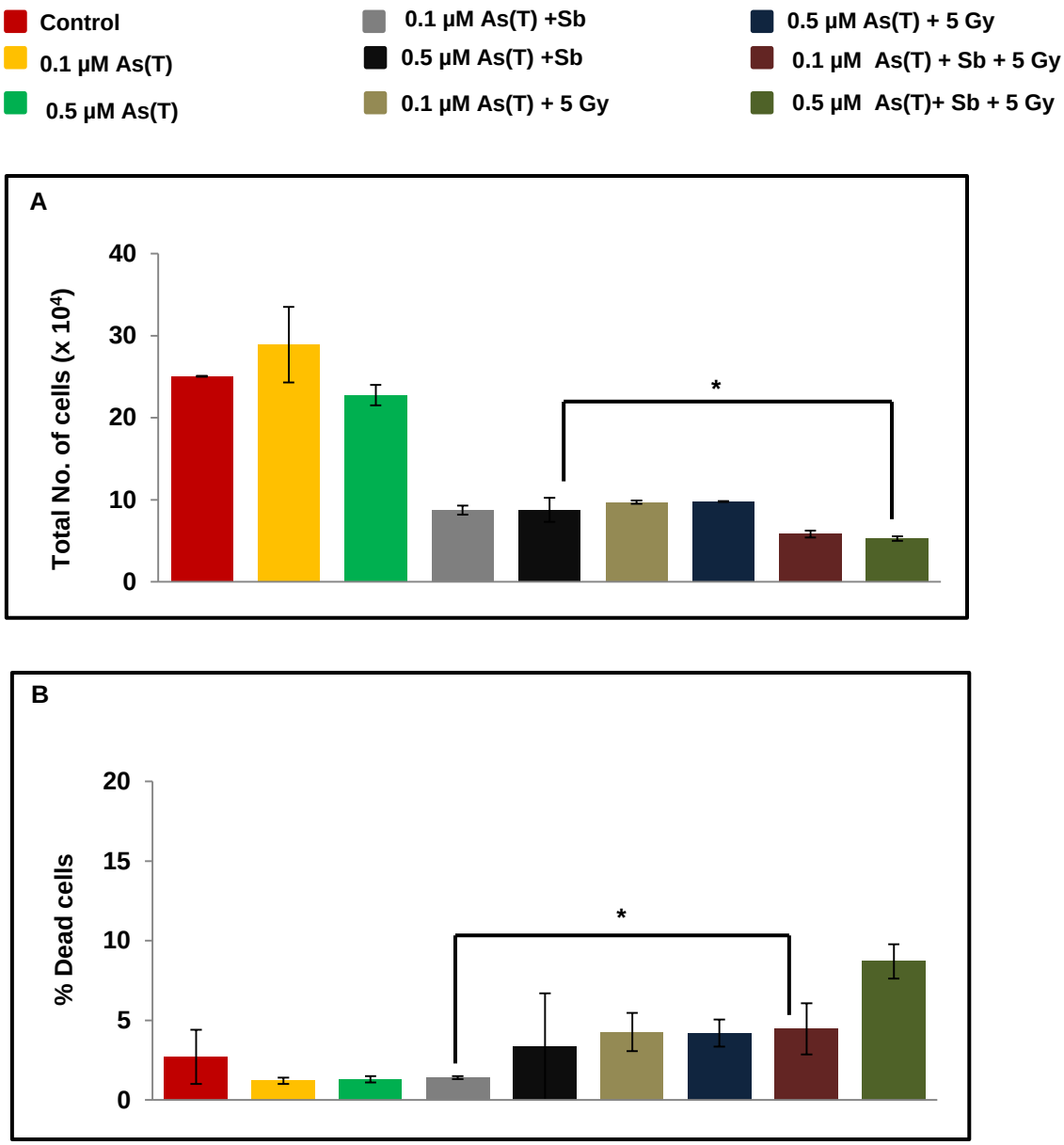


Figure 19. Effect of Silibinin and gamma radiation on HaCaT cells exposed to arsenic for one month. Transformed HaCaT cells, exposed to 0.1 and 0.5 µM concentration of arsenic, grown in 24-well plates for 24 h. Cells were treated with silibinin and exposed to 5 Gray (Gy), alone and in combination. Trypan blue assay was performed after 48 h to evaluate total number of cells and percent of dead cells as described in materials and methods. (A) Quantitative data are shown for the total number of cells in arsenic- transformed cells alone and in combination with silibinin and radiation after 48 h. (B) Quantitative data are shown for percent dead cells in arsenic transformed cells alone and in combination with silibinin and radiation after 48 h. Data are shown as mean ± SE of three independent wells and is representation of 1 independent experiments. P value: $p<0.05$ (*) . As(T): Arsenic Transformed HaCaT cells exposed to arsenic for one month, Sb: Silibinin, Fs: Fisetin

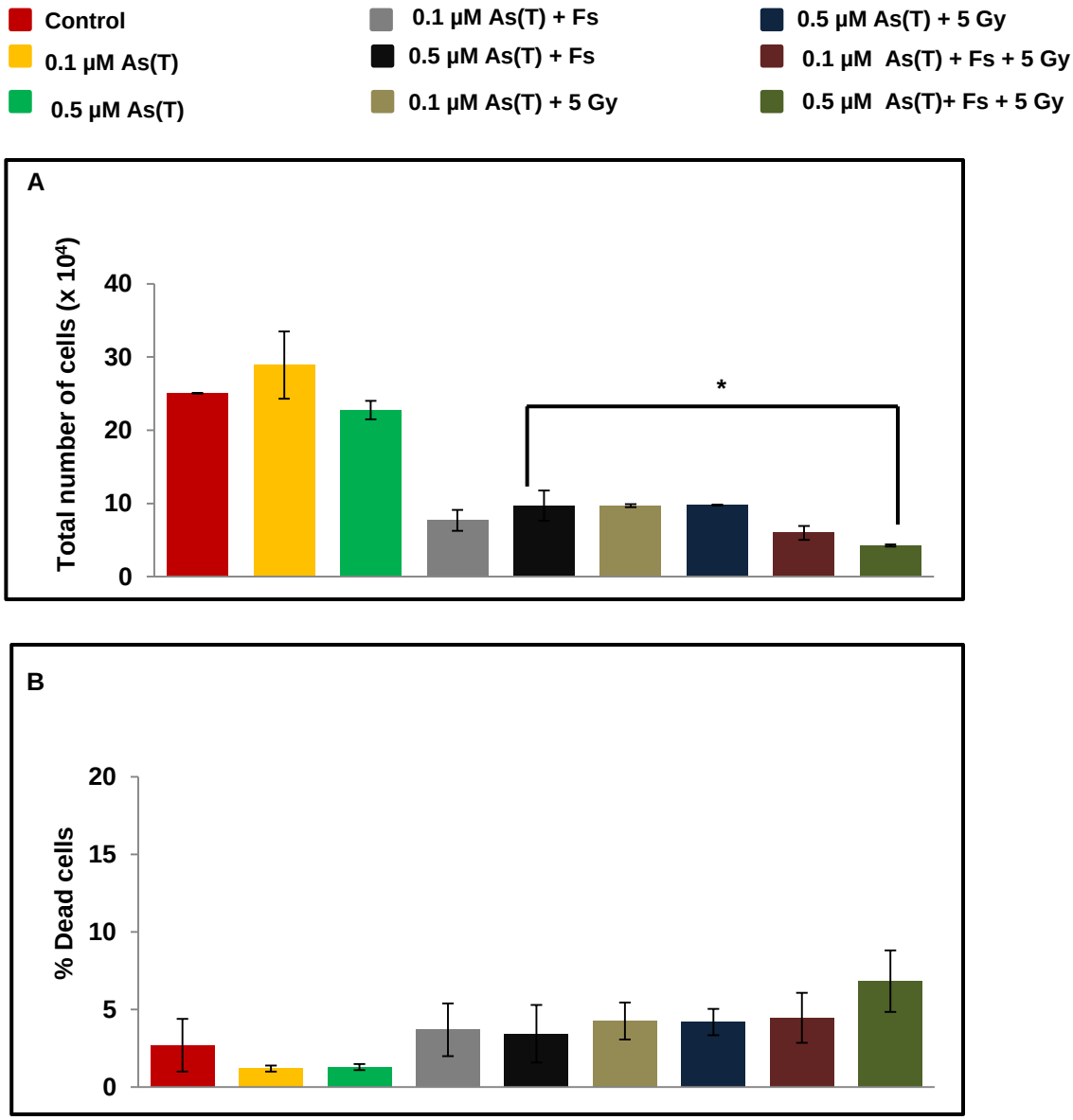


Figure 20. Effect of fisetin and gamma radiation on HaCaT cells exposed to arsenic for one month. Transformed HaCaT cells, exposed to 0.1 and 0.5 µM concentration of arsenic, grown in 24-well plates for 24 h. Cells were treated with fisetin and exposed to 5 Gray (Gy), alone and in combination. Trypan blue assay was performed after 48 h to evaluate total number of cells and percent of dead cells as described in materials and methods. (A) Quantitative data are shown for the total number of cells in arsenic- transformed cells alone and in combination with fisetin and radiation after 48 h. (B) Quantitative data are shown for percent dead cells in arsenic -transformed cells alone and in combination with fisetin and radiation after 48 h. Data are shown as mean ± SE of three independent wells and is representation of 1 independent experiments. P value: $p<0.05$ (*) . As(T): Arsenic Transformed HaCaT cells exposed to arsenic for one month, Sb: Silibinin, Fs: Fisetin

while the combination effect of fisetin and gamma radiation resulted in 79% reduction in total cell number as compared to exposed HaCaT cells at 0.1 µM alone **(Fig.20 A)**. Similarly, treatment of silibinin in 0.5 µM exposed HaCaT cells resulted in 56% inhibition in cell number while in combination with radiation resulted in 83% reduction in total number of cell as compared to exposed HaCaT cells at 0.5 µM alone **(Fig.20 A)**. In terms of cell death, combination of silibinin and radiation resulted in 7% cell death in 0.5 µM exposed HaCaT cells, as compared to 3% cell death in silibinin alone in 0.5 µM exposed HaCaT cells **(Fig.20 B)**. Overall, these results suggest that gamma radiation in combination with phytochemicals is more effective in killing transformed cells rather than alone treatment.

5.11. Effect of combination of gamma radiation and phytochemicals on morphology of transformed HaCaT cells

Combined effect of gamma radiation and phytochemicals on the morphology of transformed HaCaT cell was observed. Treatment of silibinin (100 µM) and radiation (5 Gy) in combination changed the shape of the HaCaT cells form normal cuboidal shape to round or spindle shape with less packed, with combination of arsenic dose of 0.1 and 0.5 µM at 24 hours (**Fig.21 A.3 and B.3)**. Similarly, treatment of fisetin (25 µM) and radiation (5 Gy) changed the shape of the HaCaT cells form normal cuboidal shape to round or spindle shape with less packed, at arsenic dose of 0.1 and 0.5 µM at 24 hours (**Fig.21 A.4 and B.4)**. Effect of gamma radiation alone on exposed HaCaT cells were also observed without phytochemicals treatment, but effect was less severe than combination effect (**Fig.21 A.2 and B.2)**. These morphological changes on combined treatment suggest that combination of phytochemical and radiation caused a decrease in overall number of adherent cells, and thus, inhibited the proliferation.

5.12. Increased expression of MMPs in acute and chronically exposed HaCaT cells

Till now, studies of acute and chronic effects of arsenic on HaCaT cells were done, to screen the non-cytotoxic arsenic concentration that transforms the HaCaT cells. Further, we targeted arsenic transformed HaCaT cells with phytochemical, silibinin and fisetin. Next, we explored the molecular alterations caused by acute and chronic effect of arsenic that might be involved in transformation of HaCaT cells. For that, we checked the expression of matrix metalloproteinases (MMP), using gelatin zymography, which is known to upregulate in malignant cells as compared to normal. Gelatin zymography is very sensitive method

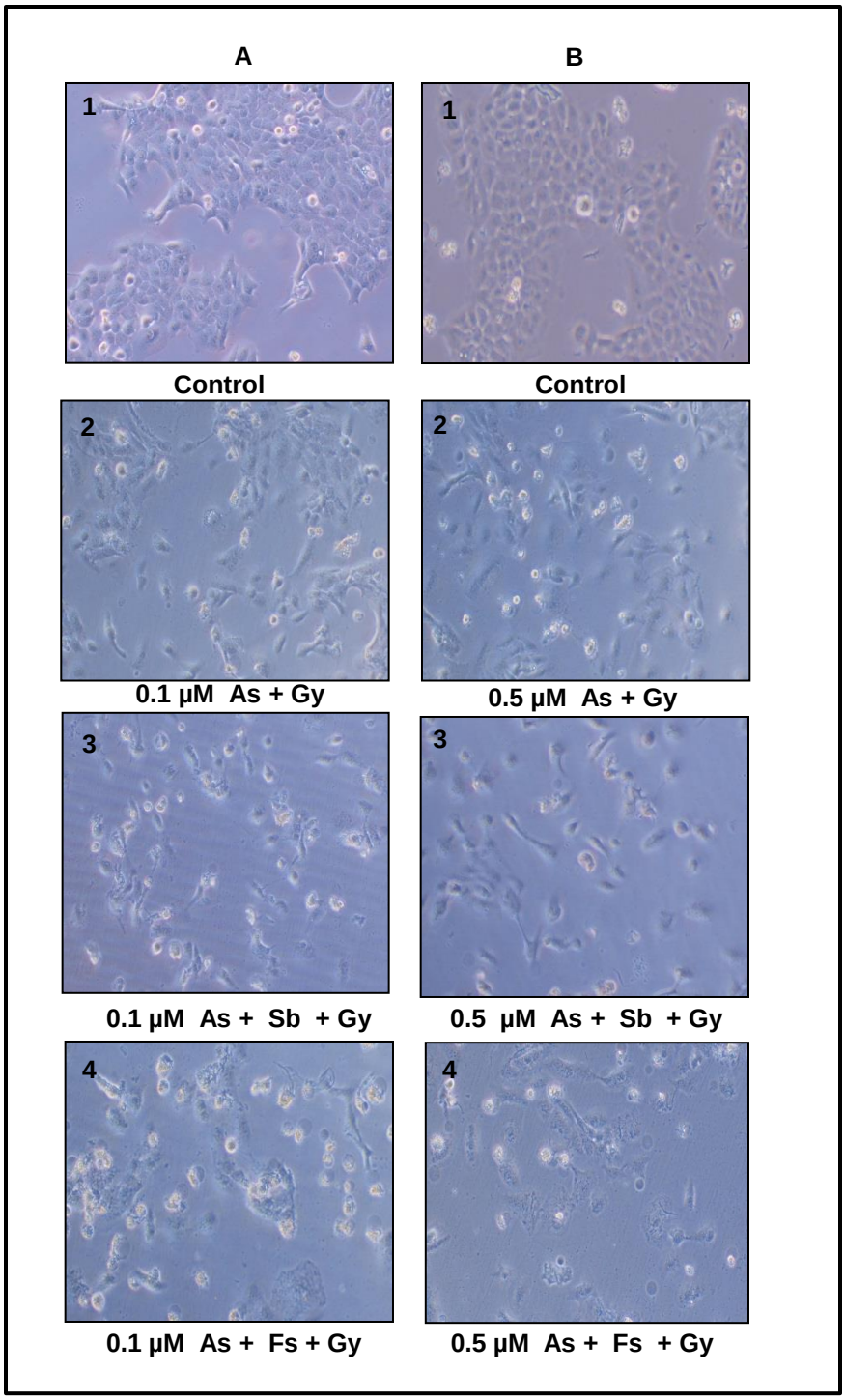


Figure 21. Effect of phytochemicals, Silibinin and Fisetin, alongwith radiation on cell morphology in the presence of arsenic. (A) Representation of morphology of HaCaT cells with 0.1 µM As and with phytochemicals and radiation, and (B) with 0.5 µM As and with phytochemicals and radiation after 24 h of treatment at 100X magnification.

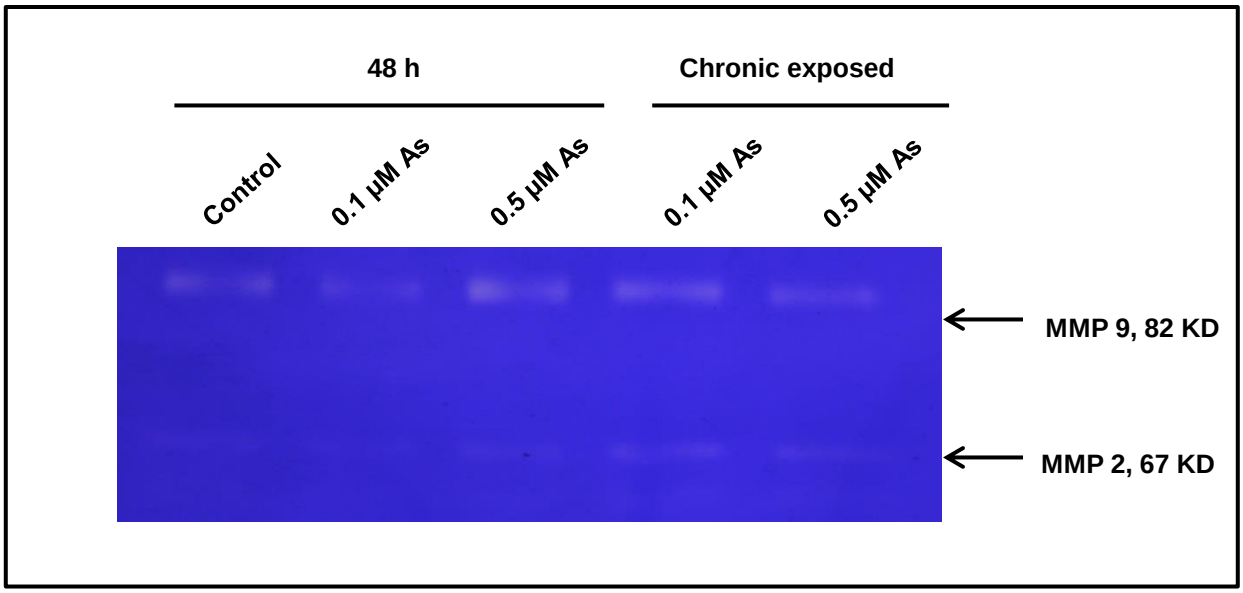


Figure 22. Effect of arsenic on matrix metalloproteinase activity. Zymography was done to asses the acute (48 h) and chronic effect of arsenic on MMPs secretion and their activity. MMPs activities were found to be elevated which can be observed from more degradation of gelatin the gel in treated as compared to control. Sb: Silibinin, Fs: Fisetin, MMP: Matrix metalloproteinases

where activity status of MMP could be detected. Therefore, we checked the modulation of expression of MMP-2 and MMP-9 caused by arsenic using gelatin zymography technique. We observed more activity of MMP-9 in HaCaT cells treated with arsenic dose of 0.5 µM for 48 hours but found to be lessen in 0.1 µM as compared to control. However, chronically exposed HaCaT cells at both doses of arsenic, 0.1 and 0.5 µM, have shown increased activity in MMP-9 secretion and activity **(Fig. 22)**. Also, there was no change seen in activity of MMP-2 at 0.1 µM dose at 48 h but a slight increased was seen at dose 0.5 µM. Further, chronically exposed HaCaT cells at both doses have shown the increased activity of the MMP-2 **(Fig. 22)**. In conclusion, upregulation of activity MMPs in HaCaT cells on treatment with arsenic indicated the possible transformation of HaCaT cells.

5.13. Acute and chronic effects of arsenic on ROS production in HaCaT cells

One of the major mechanisms, which can transform the cells in any metal induced carcinogenesis, is the production of the reactive oxygen species (ROS). Hence, we looked for the production of ROS induced by arsenic at non-cytotoxic dose. Both, acute and chronic exposed HaCaT cells were used for the experiments. DCFDA dye was used for the experiment which permeates the cells and then, inside the cell cleaved by ROS to generate fluorescence. Excitation for this dye is 485 nm and emission is 517 nm. Time kinetics study was done for looking into ROS production (5 min., 1, 3, 6, 12, 18, 24 hours). ROS was not detected in any of the treatment in time-kinetics study. Hydrogen peroxide (500 µM) was used as a positive control for the experiments and found to be significantly elevated in all the time points of the experiments **(Fig. 23)**. Since, we found a slight increase in ROS at 12 hour during the time kinetics, so we decided to detect the ROS at this time point through FACS. But, in FACS data, ROS was not detected in any of the treatments but peak of positive control, hydrogen peroxide (light blue peak) found to be shifted **(Fig. 24)**. Hence, these results suggest that at non-cytotoxic dose of arsenic, ROS might not involve in arsenic-induced transformation of cells.

5.14. Arsenic modulates expression of genes involved in transformation

To understand the molecular mechanism involved in transformation of HaCaT cells, we further looked into expression of certain genes that might involve in the arsenic induced transformation of HaCaT cell. Several genes were selected on the basis of their already known role in other cancers and differential expression in microarray studies related to transformation. So, we analysed the expression of following genes BCL2A1, IGFL1,

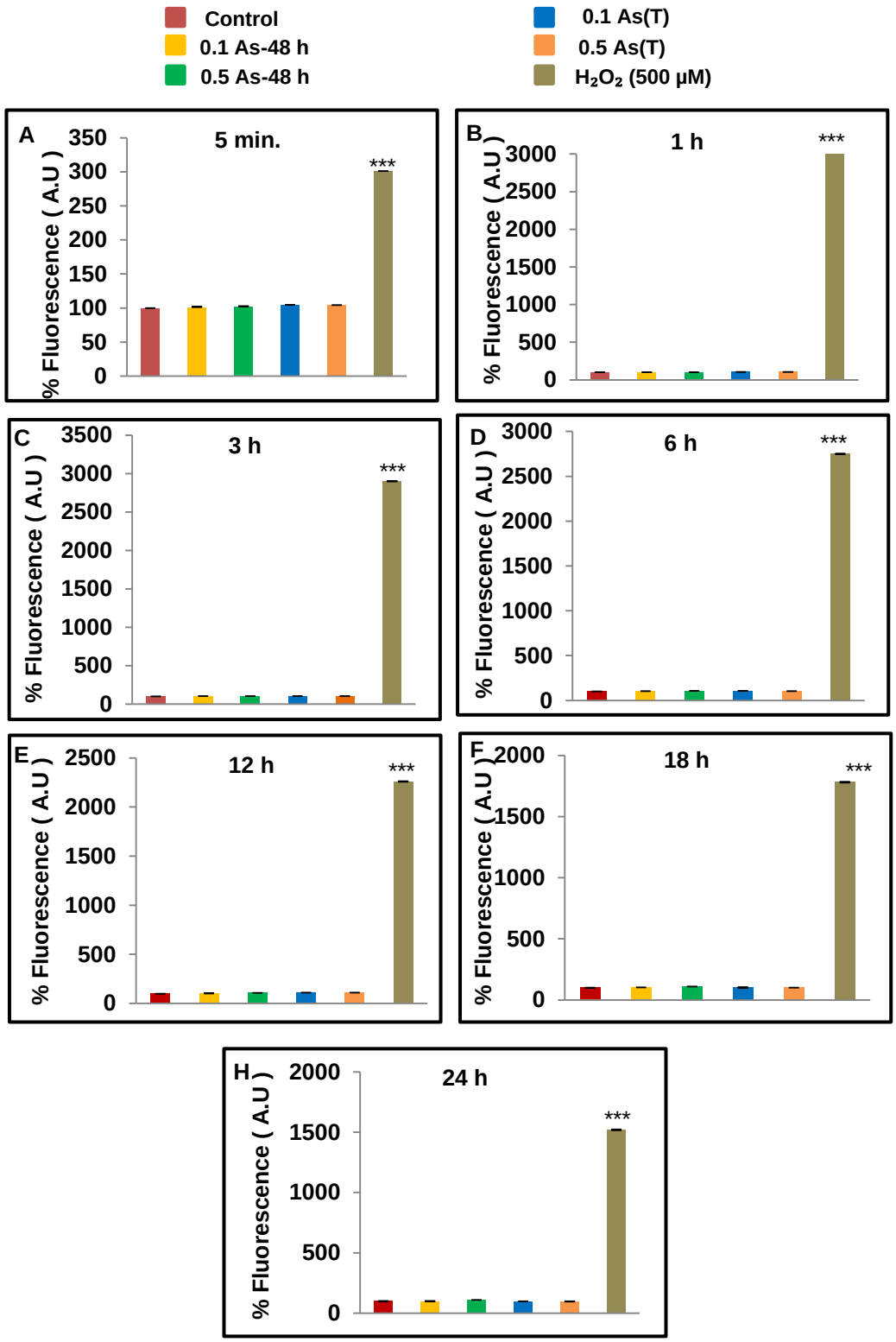


Figure 23. ROS generation time-kinetics was done using DCFDA. ROS formation upon exposure to arsenic was measured using spectrophotometer. 15 × 10^3 cells were seeded in each well of 96- well plate and grown for 24 h. DCFDA was added for 30 minutes and then respective treatment was given. At different time points, reading were taken, (5 min. 1 h, 3 h, 6 h, 12 h, 18 h and 24 h) and plotted as percent fluorescence. Hydrogen peroxide (500 µM) was used as positive control. Data are shown as mean ± SE of three independent wells and is representation of 3 independent experiments. P value: $p<0.05$ (*) . As(T): Arsenic Transformed HaCaT cells exposed to arsenic for one month, Sb: Silibinin, Fs: Fisetin, H_2O_2: Hydrogen peroxide, DCFDA: 2',7' –dichlorofluorescin diacetate

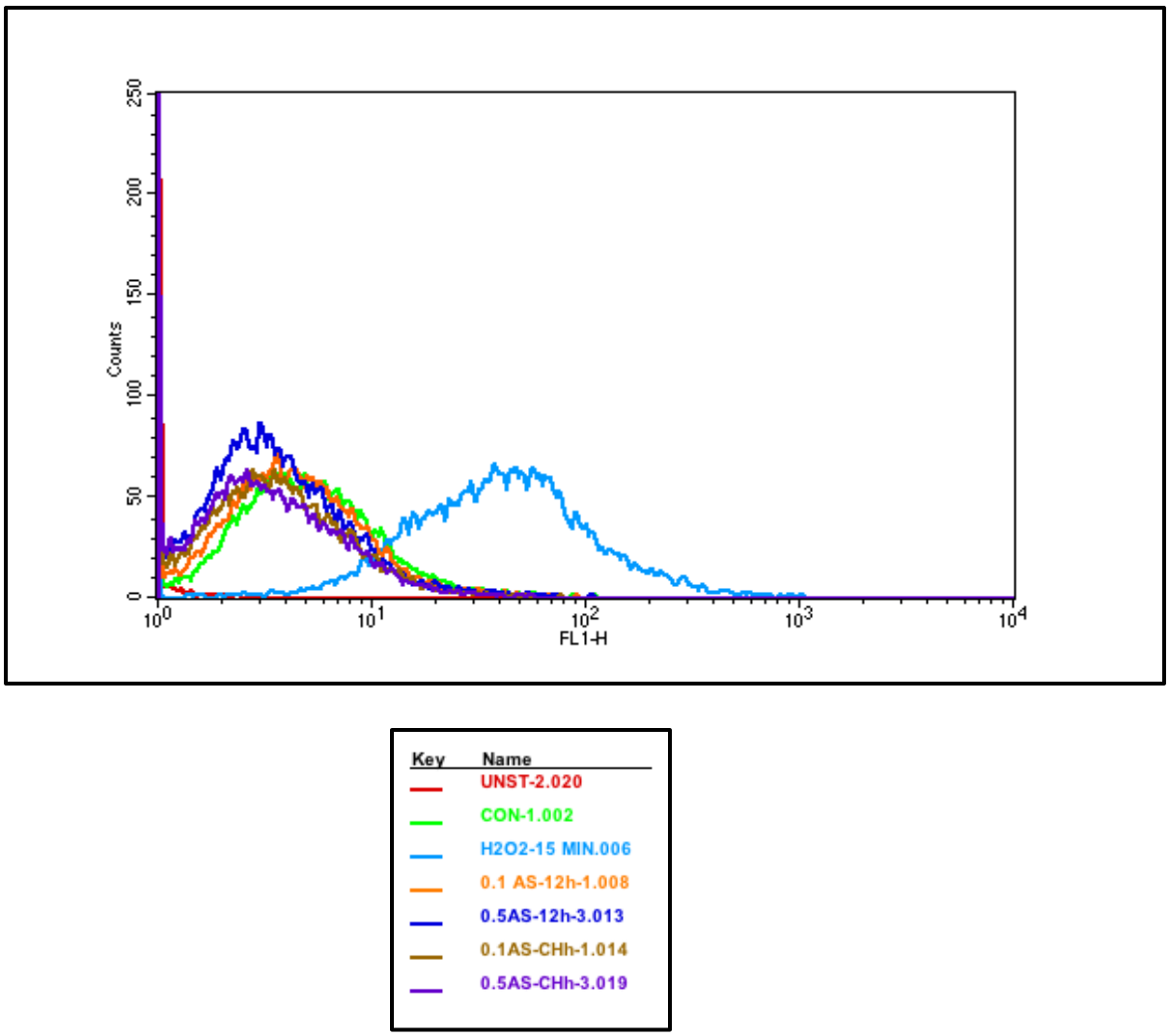


Figure 24. Measurement of ROS formation using DCFDA through flow cytometry. ROS formation upon exposure to arsenic was measured using flow cytometry at 12 h . 35×10^3 cells were seeded in each well of 24- well plate and grown for 24 h. Next day, treatment was given for 12 h. Before harvesting cells, DCFDA was added for 30 minutes. Hydrogen peroxide (500 µM) used as positive control. Data are shown as mean ± SE of three independent wells and is representation of 1 independent experiments. P value: $p<0.05$ (*). As(T): Arsenic Transformed HaCaT cells exposed to arsenic for one month, Sb: Silibinin, Fs: Fisetin, H_2O_2: Hydrogen peroxide, DCFDA: 2',7' –dichlorofluorescin diacetate

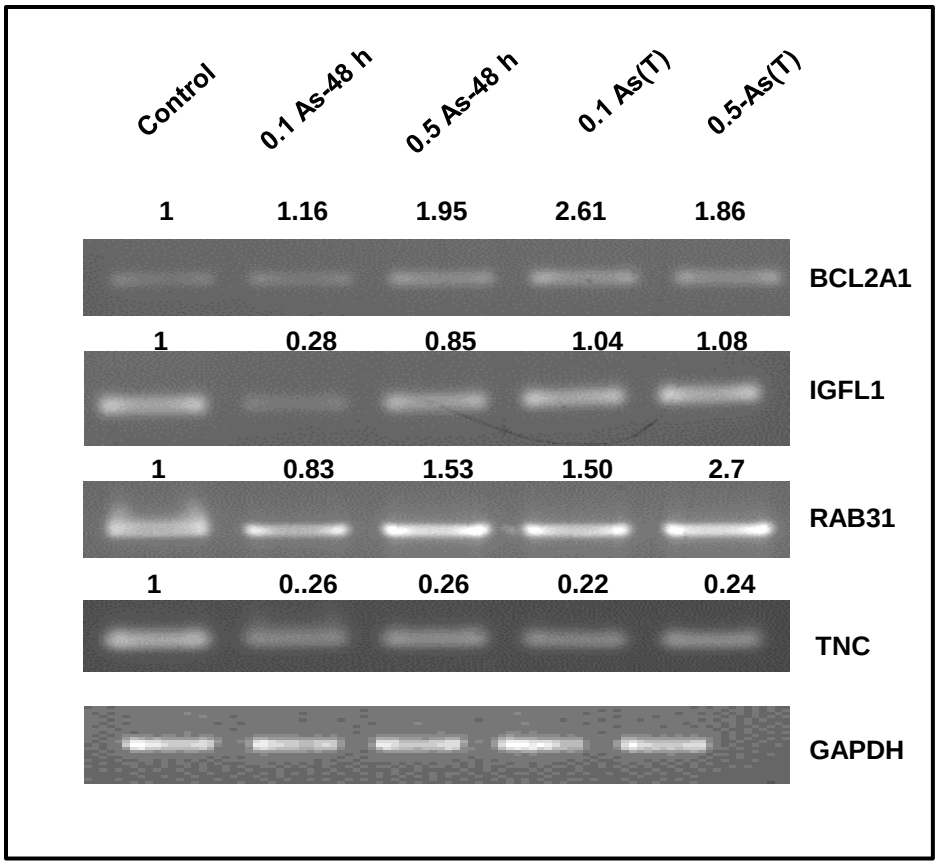


Figure 25. Arsenic upregulates the genes involved in transformation. RNA was isolated from HaCaT cells exposed to arsenic at various concentration (mention in figure) at 48 h. RT-PCR was done for expression of gene BCL2A1, IGFL1, RAB31 and TNC. GAPDH was used as loading control. Each gene was normalized with loading control and densitometry was performed for fold change.

RAB31 and TNC upon treatment with arsenic on HaCaT cells using RT-PCR. We found the upregulation of BCL2A1 expression in both acute as well as chronic treatment of arsenic at dose of 0.1 and 0.5 µM as compared to control **(Fig. 25)**. RAB31 expression was found to be elevated at 0.5 µM arsenic treatment for 48 hours but not at 0.1 µM arsenic treatment as compared to control. However, in chronically exposed HaCaT cells, RAB31 was found to be elevated in both treatments **(Fig. 25)**. TNC was found to be downregulated in acute as well as chronically exposed HaCaT cells as compared to control. Effect of arsenic on gene IGFL1 was seen only in acute treatment, where it is found to be downregulated as compared to control. The expression fold change has been depicted in the figure and GAPDH was used as a loading control **(Fig. 25)**. Changes in expression of genes associated with the treatment of arsenic in HaCaT cells could drive the transformation in HaCaT cells.

Discussion

6. Discussion

Skin cancer is one of the most common occurring cancers reported in fair-skinned population worldwide. The statistics of incidence, morbidity and mortality due to skin cancer is increasing worldwide dramatically, which poses an alarming threat to the healthcare systems (Saladi and Persaud, 2005 and Narayanan et al., 2010). According to published reports, conventionally it is considered that more melanin in the skin of Indian population has protective role in developing skin cancer, hence incidence of skin cancer is less in Indian population. Recently, it has been suggested that there is a rise of Non-melanoma skin cancer (NMSC) in India, predominantly squamous cell carcinoma (SCC) (Panda, 2010). There are several causative factors responsible for the skin cancer but ultraviolent (UV) radiation is the most common cause (Saladi and Persaud, 2005). Along with that, there are many risk factors which increase the chances of having skin cancer such as age, family history, immune system, infection from certain viruses and chemicals such as arsenic, coal tar, soot etc.

Increase in incidence rate of skin cancers during past few decades due to arsenic exposure has posed a threat to human health around the globe including India. Arsenic crisis have been reported in more than 30 countries. In India, regions in the river basin of Ganga, Brahmaputra and Meghna river are the most affected regions in the world, where arsenic contaminated water affecting around 6 million people. Severe affected regions West Bengal, in India, and Bangladesh are the most studied area where many arsenic related malignancies have been reported including skin cancer. Arsenic crisis is attributed due to dependency of large population on arsenic contaminated groundwater for their household uses (Guha Mazumder and Dasgupta, 2011). Studies have explored that the exposure of individual to arsenic rich water over a long period leads to arsenic poisoning known as arsenicosis. This poisoning affects the systematic function of many organs in the body but skin is the most commonly affected and responsive organ. However, other parts have also reported to be get affected from arsenic toxicity such as peripheral vascular system, kidney, heart, liver, lung, prostate, uterus, bladder and lymphatic tissues (Sengupta et al., 2008).

According to WHO, maximum permissible concentration for arsenic in drinking water is 10 µg/L which corresponds to 0.1 µM, but several countries including India has standardized the maximum permissible limit for arsenic in drinking water as 50 µg/L. It is reported that individual exposed to high level of arsenic generally have arsenic in their blood in range from 0.05 to 1.2 µM (Snow et al., 2005). Research with low concentrations

of arsenic is very controversial, since studies have not been able to find out the threshold of arsenic concentration below that arsenic is not harmful but some researchers believed that no such threshold exist for arsenic. So, it is important to explore the effect of low concentrations of arsenic and its associated mechanisms. Therefore, in present study, we have studied the effect of lower concentrations of arsenic on HaCaT cells, skin keratinocytes, to better understand the arsenic induced skin carcinogenesis at low concentrations.

We have screened a range of arsenic concentrations on HaCaT cells and found increased proliferation in HaCaT cells at low doses of arsenic 0.05, 0.1 and 0.5 at 48 and 96 hours, as compared to control as assessed by MTT and trypan blue assay. Concentration of arsenic >0.5 μM was found to be toxic to the cells. The result was found to be consistent with published work where similar proliferation was seen at 0.1 and 0.5 μM concentration of arsenic at 3 and 5 days of treatment (Zhang et al., 2003). Serum-starvation is the method that modifies and induces dynamic response in the cells, and thus affects the outcomes of the experiment (Pirkmajer and Chibalin, 2011). We have tried to explore the effect of arsenic on serum-starved HaCaT cells (grown in 1% FBS), but, similar effects were seen with arsenic treatment as found with HaCaT cells grown in regular conditions. Effect of low to high concentrations of arsenic on clonogenic potential of HaCaT cells was assessed using clonogenic assay and results were found to have implications in toxicity of arsenic. The results of clonogenic assay suggest that low doses of arsenic are non-toxic to the cell, while doses greater than 0.5 μM are toxic to the HaCaT cells. But, the another significant point need to discuss here is that the range of low dose of arsenic is modifiable, some literature studies have shown that low concentration of arsenic could be considered from range 0.1-1 μM (Zhang et al., 2003 and Wu et al., 2012) while in another study low dose of arsenic is considered upto 2 μM (Qin et al., 2008). From our study, we can conclude that arsenic concentrations upto 0.5 μM are non-cytotoxic, therefore, we have taken those concentrations which are low as well a non-cytotoxic to the HaCaT cells.

Further, we examined the transforming ability of arsenic at low, non-cyotoxic concentration on HaCaT cells (0.1 and 0.5 μM). Several studies have reported malignant transformation of HaCaT cells on chronic exposure to arsenic for varying time points. In one study, HaCaT cells exposed to 0.05 μM arsenic for 25 passages caused malignant transformation in cells with visible morphological changes (Huang et al., 2013). Chronic effect of arsenic at dose 0.1 μM on HaCaT cells for 14 days have shown many cell cycle related effects. Further,

exposure with arsenic dose 0.1 μM for 28 weeks resulted in resistance to apoptosis in transformed HaCaT cells induced by UV irradiation and chemotherapeutic compounds (Liao et al., 2011). In our study, we exposed the HaCaT cells with arsenic at low, non-cytotoxic dose (0.1 and 0.5 μM) for 1 month and cells were found to be transformed as confirmed by soft agar colony formation assay. We have used A431, skin cancer cells, as positive control where many colonies were formed on soft agar. Thus, we have observed acute as well as chronic effects of arsenic on transformation of HaCaT cells. Further, we targeted acute and chronically exposed arsenic induced transformed HaCaT cells by using phytochemicals which have shown anticancer potential.

Chemoprevention is a novel approach used to target or prevent carcinogenesis using natural or synthetic products. Almost, all these compounds possess anticancer, anti-mutagenic and antioxidants properties. Many of these compounds have been studied with respect to skin carcinogenesis and found to be beneficial. Since, most of the studies for phytochemicals have been done at high concentrations of arsenic with aim to lessen the toxic effect of arsenic. In present study, we have used phytochemicals, silibinin and fisetin, to target transformed HaCaT cells induced through non-cytotoxic dose of arsenic. We have found strong-inhibition of arsenic induced proliferation in HaCaT cells upon treatment of silibinin with 100 μM and fisetin with 25 μM. Similar effects of phytochemicals were seen on acute as well as chronically exposed HaCaT cells. Also, morphological changes in HaCaT cells were observed with phytochemical treatment. Phytochemicals treated cells were found to be more round in shape with less number of adherent cells. Many studies have shown the effective synergistic effect of phytochemicals with radiation (Hazra, et al., 2011). We have used gamma radiation in combination with phytochemicals, silibinin or fisetin on chronically exposed HaCaT cells. We observed more inhibition in combination of radiation and phytochemical as compared to their alone treatments. Phytochemicals have been reported to show cell cycle arrest in transformed HaCaT cells assessed through flow cytometry. Silibinin showed G1 and G2/M arrest while fisetin have shown G2/M arrest in HaCaT transformed cells. Overall, these phytochemicals could be investigated further to evaluate their mitigating effect on individuals exposed to arsenic contaminated water.

To assess the reversibility of the chronic effect of arsenic on the HaCaT cells, we have taken cryopreserved vial and revived it and then, grown transformed HaCaT cells for some passages in arsenic-free media. Then, we studied the growth kinetics of these transformed cells. We observed higher rate of proliferation in transformed HaCaT cells at concentration

of 0.1 μM while it was not observed for arsenic concentration of 0.5 μM transformed HaCaT cells. The reason could be that chronic exposure of low, non-cyotoxic 0.5 μM dose of arsenic might be toxic to the HaCaT cells, further, and it might induce stress to the cells and causing genomic unstability in cells.

Furthermore, we tried to explore the molecular alterations associated with acute and chronic exposure of arsenic involved in transformation of HaCaT cells. Effect of arsenic on HaCaT cells was found to be concentration-dependent. Arsenic exerts its effect through genotoxic as well as non-genotoxic mechanisms in skin carcinogenesis. Arsenic and its metabolites cause several molecular alterations such as oxidative stress, chromosomal aberrations, antioxidants enzyme, DNA repair enzymes and DNA hypomethylation and these changes are associated with transformation (Rossman, 2003). Also, several cell cycles regulatory molecules such as p53 and p21 have been found to be affected by treatment (Rossman, 2003).

MMPs are important proteins for tumor growth and progression and their unregulated expression is reported to be associated with various diseases including cancer. Secretion of MMPs is usually increased in cancer to degrade extracellular matrix. Effects of arsenic on MMPs secretions usually have been found to be associated with malignant transformation of cells. Several studies have shown increased expression of MMPs upon treatment with arsenic (Benbrahim-Tallaa et al., 2005 and Pi et al., 2008). It has been reported that the treatment of HaCaT cells with 0.1 μM dose of arsenic for 20 weeks results in malignant transformation of HaCaT cells with increase secretion of active form of MMP-9 as compared to control (Sun et al., 2009). In another study, it was found that the treatment of HaCaT cells with 0.1 μM dose of arsenic for 28 weeks resulted in increased activity of active form of MMP-9 but no change was found in MMP-2 (Pi et al., 2008). In our study, we have evaluated the acute as well chronic effect of arsenic on secretions of MMPs in HaCaT cells. We observed an increased activity of MMP-9 in acute as well as chronically exposed HaCaT cells. The effect of arsenic at dose 0.1 μM on MMP-9 and MMP-2 activity was found negligible as compared to control in HaCaT cells at 48 hours. However, acute exposure of 0.5 μM arsenic for 48 hours and chronic exposed HaCaT cells at 0.1 μM as well as 0.5 μM resulted in increased MMP-2 activity in HaCaT cells as compared to untreated cells of same passage number. Hence, increased active MMPs activity upon treatment with non-cytotoxic, low concentrations of arsenic might be one of the important molecular events involved in transformation of HaCaT cells.

Reactive oxygen species (ROS) are important for maintaining the homeostasis of the cell. It is reported that production of ROS due to arsenic is dependent on cell type, as more ROS formation found in HaCaT cells as compared to fibroblast cells upon arsenic treatment. HaCaT cells exposed to 0.1 μM of arsenic dose for 28 weeks have shown no changes in ROS formation as compared to control (Pi et al., 2005). Even, HaCaT cells gown at arsenic dose at 1 μM for 14 passages have not shown any obvious change in ROS formation as compared to control, but after 14 passages ROS level gradually declined compared to untreated (Ma et al., 2015). Consistent with these results, we observed no ROS production in acute as well as chronically exposed HaCaT cells. Time-kinetics studies for 24 hours have not shown any increase in ROS production upon arsenic treatment. Further, ROS was also measured through flow cytometry to support these finding. Hence, it can be concluded that at low, non-cytotoxic concentrations of arsenic might not involve ROS in transformation of HaCaT cells. There could be other mechanisms involved in transformation of HaCaT cells at low doses of arsenic.

Further, we looked into the effect of arsenic on expression of genes that might be involved in the transformation of HaCaT cells and these genes include BCL2A1, IGFL1, RAB31 and TNC. BCL2A1 (B-cell lymphoma 2-related protein A1) is an important apoptosis regulatory protein whose expression has found to be increased in many cancer. Altered expression of BCL2A1 is found associated with transformation of cells and also involved oncogenic transformation in melanocytes (Vogler, 2012 and Haq et al., 2013). IGFL1 are IGF-like 1 gene likely act as growth regulators, found to be overexpressed in benign fraction as compared to malignant fraction of prostate cancer (Birnie et al., 2008). RAB 31 is small GTPases protein involved in intercellular trafficking, which is related to highly regulated process of cell proliferation and migration. This protein is found to be dysregulated in several cancers, and therefore, abnormal expression is associated with transformation of cells with signaling modulation (Chua and Tang, 2015). Tenascin-C gene is a matricelullar protein which regulates the cell adhesion, differentiation, migration and growth through modulating the interactions of cell with extracellular matrix and growth factors**.** This gene is found to be dysregulated in cancer and promotes invasion, with their role in transformation in rat embryonic fibroblasts through de-adhesion (Chiovaro et al., 2015). We have studied the expression of all these genes in HaCaT cells upon arsenic treatment. We found increased expression of BCL2A1 and RAB31 in all acute and chronic treatment of arsenic while TNC expression was found to be decreased as compared to

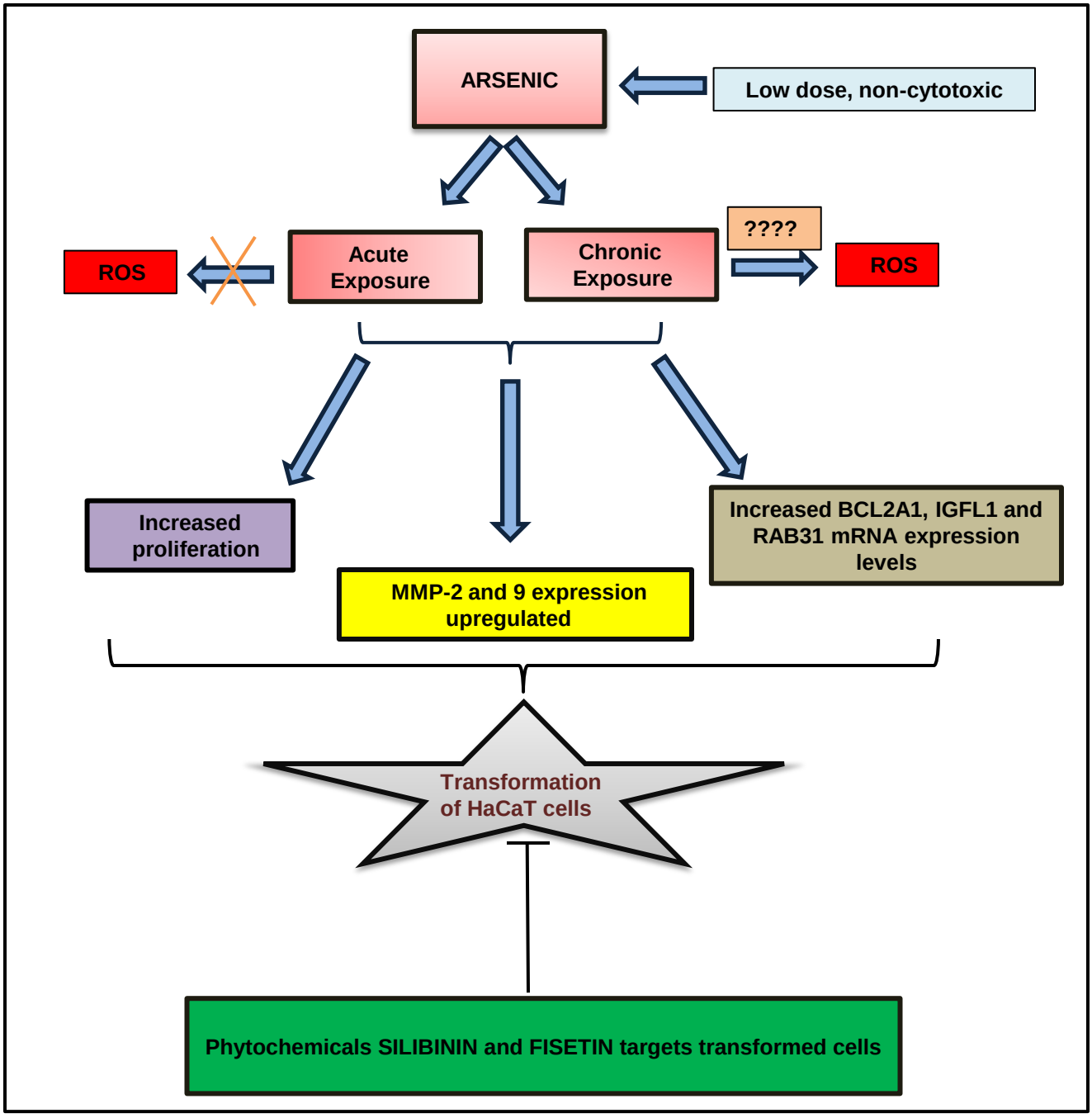


Figure 26. Schematic flow-chart showing various molecular events involved in arsenic-induced transformation of HaCaT cells. Both acute as well as chronic exposure of arsenic causes molecular alterations in HaCaT cells. Non–cytotoxic doses of arsenic causes increased proliferation, enhanced MMP-2 and 9 activity and upregulation of genes that might involved in transformation. Role of ROS in arsenic induced transformation of HaCaT cells at low dose is not clear. Transformed HaCaT cells can be targeted through phytochemicals, silibinin and fisetin.

control. IGFL1 expression was decreased at 0.1 μM dose of arsenic while at other doses no changes were observed.

In conclusion, our study has explored the acute as well chronic effect of arsenic on the HaCaT cells at low, non-toxic concentrations **(Fig.26).** In this study, we tried to understand the mechanisms associated with arsenic-induced transformation of HaCaT cells. We have found several molecular alterations upon arsenic treatment such as altered MMPs expression and change in expression of certain genes including BCL2A1, TNC, RAB31 and IGFL1. These molecular changes could involve in transformation of HaCaT cells. Further, arsenic-induced transformed HaCaT cells could be targeted using phytochemicals, silibinin and fisetin.

Summary and Conclusion

7. Summary and conclusion

Skin cancer is the most common occurring cancer worldwide including India. Several factors are responsible for skin cancer in humans, but major factor is UV radiation from sunlight. However, the incidence of arsenic-induced skin cancer is increasing worldwide due to dependency of millions of people on arsenic contaminated drinking ground water. It has been reported that low doses of arsenic is found in blood of individuals exposed to arsenic for a long period time. In present study, we have studied the effect of non-toxic, low concentrations of arsenic on HaCaT cells, which represent a human skin keratinocytes.

We have found that low doses of arsenic induced proliferation and transformation in the HaCaT cells. The low doses were found to be non-toxic to the HaCaT cells. We have explored the both acute as well as chronic effect of low doses of arsenic on cells. Acute exposure of arsenic too has shown proliferation in HaCaT cells, which might indicate the early transformation capability of arsenic at low doses. Further, we used phytochemicals, silibinin and fisetin to target arsenic-induced transformed cells, and observed reduced effect of acute and chronic exposure of arsenic on HaCaT cells.

It is important to explore the molecular mechanisms associated with arsenic-induced transformation of HaCaT cells caused by low doses of arsenic. We have looked into several molecular alterations caused by arsenic on HaCaT cells during the process of transformation. We have found increased activity of active form of MMP-9 and MMP-2 in arsenic treatments. Thereafter, we measured the ROS level induced by arsenic at low doses, but, ROS level did not alter during aresnic treatment. Further, we studied the acute as well as chronic effect of arsenic on genes those have known role in transformation in other cancer types. We have found increased expression of BCL2A1 and RAB31 in both acute as well as chronic treatment of arsenic. However, expression of gene TNC found to be decreased. These altered gene expressions might have been involved in arsenic-induced transformation in HaCaT cells. Hence, it is needed to further explore the effects of arsenic on these genes and associated molecular signaling.

In future perspectives, we further need to explore the effect of arsenic at non-toxic low concentrations of arsenic on HaCaT cells and associated mechanisms involved in transformation. The deciphering of mechanisms could help in providing insight in arsenic-mediated skin carcinogenesis in population who are exposed to arsenic continuously through drinking water. This would also help in finding preventive measures as observed in

the present study where non-toxic phytochemicals or flavonoids such as silibinin and fisetin inhibited the arsenic-induced transformation as well as growth of skin keratinocytes.

References

8. References

- Adhami, V.M., Syed, D.N., Khan, N., and Mukhtar, H. (2012). Dietary flavonoid fisetin: a novel dual inhibitor of PI3K/Akt and mTOR for prostate cancer management. Biochemical pharmacology *84*, 1277-1281.
- Agarwal, C., Singh, R.P., Dhanalakshmi, S., Tyagi, A.K., Tecklenburg, M., Sclafani, R.A., and Agarwal, R. (2003). Silibinin upregulates the expression of cyclin-dependent kinase inhibitors and causes cell cycle arrest and apoptosis in human colon carcinoma HT-29 cells. Oncogene *22*, 8271-8282.
- Agarwal, R., Katiyar, S.K., Khan, S.G., and Mukhtar, H. (1993). Protection against ultraviolet B radiation-induced effects in the skin of SKH-1 hairless mice by a polyphenolic fraction isolated from green tea. Photochemistry and photobiology *58*, 695-700.
- Ali, I., Wani, W.A., and Saleem, K. (2011). Cancer scenario in India with future perspectives. Cancer therapy *8*, 56-70.
- Anand, P., Kunnumakkara, A.B., Sundaram, C., Harikumar, K.B., Tharakan, S.T., Lai, O.S., Sung, B., and Aggarwal, B.B. (2008). Cancer is a preventable disease that requires major lifestyle changes. Pharmaceutical research *25*, 2097-2116.
- Andrew, A.S., Karagas, M.R., and Hamilton, J.W. (2003). Decreased DNA repair gene expression among individuals exposed to arsenic in United States drinking water. International journal of cancer *104*, 263-268.
- Antman, K.H. (2001). Introduction: the history of arsenic trioxide in cancer therapy. The oncologist *6*, 1-2.
- Aposhian, H.V., and Aposhian, M.M. (2006). Arsenic toxicology: five questions. Chemical research in toxicology *19*, 1-15.
- Bardach, A.E., Ciapponi, A., Soto, N., Chaparro, M.R., Calderon, M., Briatore, A., Cadoppi, N., Tassara, R., and Litter, M.I. (2015). Epidemiology of chronic disease related to arsenic in Argentina: A systematic review. The Science of the total environment *538*, 802-816.
- Basu, A., Mahata, J., Gupta, S., and Giri, A.K. (2001). Genetic toxicology of a paradoxical human carcinogen, arsenic: a review. Mutation research *488*, 171-194.
- Baudouin, C., Charveron, M., Tarroux, R., and Gall, Y. (2002). Environmental pollutants and skin cancer. Cell biology and toxicology *18*, 341-348.
- Benbrahim-Tallaa, L., Waterland, R.A., Styblo, M., Achanzar, W.E., Webber, M.M., and Waalkes, M.P. (2005). Molecular events associated with arsenic-induced malignant transformation of human prostatic epithelial cells: aberrant genomic DNA

methylation and K-ras oncogene activation. Toxicology and applied pharmacology *206*, 288-298.

- Benramdane, L., Accominotti, M., Fanton, L., Malicier, D., and Vallon, J.J. (1999). Arsenic speciation in human organs following fatal arsenic trioxide poisoning--a case report. Clinical chemistry *45*, 301-306.
- Bhattacharya, P., Samal, A.C., Majumdar, J., and Santra, S.C. (2010). Accumulation of arsenic and its distribution in rice plant (Oryza sativa L.) in Gangetic West Bengal, India. Paddy and Water Environment *8*, 63-70.
- Bode, A.M., and Dong, Z. (2002). The paradox of arsenic: molecular mechanisms of cell transformation and chemotherapeutic effects. Critical reviews in oncology/hematology *42*, 5-24.
- Burns, F.J., Uddin, A.N., Wu, F., Nadas, A., and Rossman, T.G. (2004). Arsenic-induced enhancement of ultraviolet radiation carcinogenesis in mouse skin: a dose-response study. Environ Health Perspect *112*, 599-603.
- Calatayud, M., Barrios, J.A., Velez, D., and Devesa, V. (2012). In vitro study of transporters involved in intestinal absorption of inorganic arsenic. Chemical research in toxicology *25*, 446-453.
- Carew, M.W., Naranmandura, H., Shukalek, C.B., Le, X.C., and Leslie, E.M. (2011). Monomethylarsenic diglutathione transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Drug metabolism and disposition: the biological fate of chemicals *39*, 2298-2304.
- Centeno, J.A., Mullick, F.G., Martinez, L., Page, N.P., Gibb, H., Longfellow, D., Thompson, C., and Ladich, E.R. (2002). Pathology related to chronic arsenic exposure. Environmental Health Perspectives *110*, 883.
- Chen, P.H., Lan, C.C.E., Chiou, M.H., Hsieh, M.C., and Chen, G.S. (2005). Effects of arsenic and UVB on normal human cultured keratinocytes: impact on apoptosis and implication on photocarcinogenesis. Chemical research in toxicology *18*, 139-144.
- Chou, RH., Hsieh, S.C., Yu, Y.L., Huang, M.H., Huang, Y.C., and Hsieh, Y.H. (2013). Fisetin inhibits migration and invasion of human cervical cancer cells by down-regulating urokinase plasminogen activator expression through suppressing the p38 MAPK-dependent NF-κB signaling pathway. PloS one *8*, e71983.
- Cui, X., Wakai, T., Shirai, Y., Hatakeyama, K., and Hirano, S. (2006). Chronic oral exposure to inorganic arsenate interferes with methylation status of p16INK4a and

RASSF1A and induces lung cancer in A/J mice. Toxicological Sciences *91*, 372-381.

- Dhanalakshmi, S., Mallikarjuna, G., Singh, R.P., and Agarwal, R. (2004). Silibinin prevents ultraviolet radiation-caused skin damages in SKH-1 hairless mice via a decrease in thymine dimer positive cells and an up-regulation of p53-p21/Cip1 in epidermis. Carcinogenesis *25*, 1459-1465.
- Druwe, I.L., and Vaillancourt, R.R. (2010). Influence of arsenate and arsenite on signal transduction pathways: an update. Archives of toxicology *84*, 585-596.
- Evens, A.M., Tallman, M.S., and Gartenhaus, R.B. (2004). The potential of arsenic trioxide in the treatment of malignant disease: past, present, and future. Leukemia research *28*, 891-900.
- Fatmi, Z., Azam, I., Ahmed, F., Kazi, A., Gill, A.B., Kadir, M.M., Ahmed, M., Ara, N., and Janjua, N.Z. (2009). Health burden of skin lesions at low arsenic exposure through groundwater in Pakistan. Is river the source? Environmental research *109*, 575-581.
- Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., and Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer *136*, E359-386.
- Florea, A.M., and Büsselberg, D. (2013). The two opposite facets of arsenic: toxic and anticancer drug. Journal of Local and Global Health Science, 1.
- Florea, A.M., and Busselberg, D. (2009). Anti-cancer drugs interfere with intracellular calcium signaling. Neurotoxicology *30*, 803-810.
- Freeman, G.B., Schoof, R.A., Ruby, M.V., Davis, A.O., Dill, J.A., Liao, S.C., Lapin, C.A., and Bergstrom, P.D. (1995). Bioavailability of arsenic in soil and house dust impacted by smelter activities following oral administration in cynomolgus monkeys. Fundamental and applied toxicology : official journal of the Society of Toxicology *28*, 215-222.
- Frith, J. (2013). Arsenic – the "Poison of Kings" and the "Saviour of Syphilis. Journal of Military and Veterans Health, *Vol 21 No. 4*, 11-17.
- Ge, Y., Zhang, Y., Chen, Y., Li, Q., Chen, J., Dong, Y., and Shi, W. (2011). Silibinin causes apoptosis and cell cycle arrest in some human pancreatic cancer cells. International journal of molecular sciences *12*, 4861-4871.
- Germolec, D.R., Yoshida, T., Gaido, K., Wilmer, J.L., Simeonova, P.P., Kayama, F., Burleson, F., Dong, W., Lange, R.W., and Luster, M.I. (1996). Arsenic induces

overexpression of growth factors in human keratinocytes. Toxicology and applied pharmacology *141*, 308-318.

- Guha Mazumder, D., and Dasgupta, U.B. (2011). Chronic arsenic toxicity: studies in West Bengal, India. The Kaohsiung journal of medical sciences *27*, 360-370.
- Gutierrez, M., Alarcón-Herrera, M.T., and Camacho, L.M. (2009). Geographical distribution of arsenic in sediments within the Rio Conchos Basin, Mexico. Environmental Geology *57*, 929-935.
- Harrington, J.M., Middaugh, J.P., Morse, D.L., and Housworth, J. (1978). A survey of a population exposed to high concentrations of arsenic in well water in Fairbanks, Alaska. American journal of epidemiology *108*, 377-385.
- Hazra, B., Ghosh, S., Kumar, A., and Pandey, B.N. (2011). The Prospective Role of Plant Products in Radiotherapy of Cancer: A Current Overview. Frontiers in Pharmacology *2*, 94.
- Hayakawa, T., Kobayashi, Y., Cui, X., and Hirano, S. (2005). A new metabolic pathway of arsenite: arsenic-glutathione complexes are substrates for human arsenic methyltransferase Cyt19. Archives of toxicology *79*, 183-191.
- Hei, T.K., Liu, S.X., and Waldren, C. (1998). Mutagenicity of arsenic in mammalian cells: role of reactive oxygen species. Proceedings of the National Academy of Sciences *95*, 8103-8107.
- Herbert, K.J., Holloway, A., Cook, A.L., Chin, S.P., and Snow, E.T. (2014). Arsenic exposure disrupts epigenetic regulation of SIRT1 in human keratinocytes. Toxicology and applied pharmacology *281*, 136-145.
- Hughes, M.F., Beck, B.D., Chen, Y., Lewis, A.S., and Thomas, D.J. (2011a). Arsenic exposure and toxicology: a historical perspective. Toxicological sciences : an official journal of the Society of Toxicology *123*, 305-332.
- Hughes, M.F., Beck, B.D., Chen, Y., Lewis, A.S., and Thomas, D.J. (2011b). Arsenic exposure and toxicology: a historical perspective. Toxicological Sciences *123*, 305-332.
- Jain, C.K., and Ali, I. (2000). Arsenic: occurrence, toxicity and speciation techniques. Water Research *34*, 4304-4312.
- Jennrich, P. (2013). The Influence of Arsenic, Lead, and Mercury on the Development of Cardiovascular Diseases. ISRN Hypertension *2013*, 15.
- Jing, Y. (2004). The PML-RARalpha fusion protein and targeted therapy for acute promyelocytic leukemia. Leukemia & lymphoma *45*, 639-648.

- Jomova, K., Jenisova, Z., Feszterova, M., Baros, S., Liska, J., Hudecova, D., Rhodes, C.J., and Valko, M. (2011). Arsenic: toxicity, oxidative stress and human disease. Journal of applied toxicology *31*, 95-107.
- Kenyon, E.M., and Hughes, M.F. (2001). A concise review of the toxicity and carcinogenicity of dimethylarsinic acid. Toxicology *160*, 227-236.
- Khan, N., Syed, D.N., Ahmad, N., and Mukhtar, H. (2013). Fisetin: a dietary antioxidant for health promotion. Antioxidants & redox signaling *19*, 151-162.
- Kitchin, K.T. (2001). Recent advances in arsenic carcinogenesis: modes of action, animal model systems, and methylated arsenic metabolites. Toxicology and applied pharmacology *172*, 249-261.
- Kumar, A., Kumar, V., and Kumar, A., (2015). Arsenic Distribution in Groundwater and its Effect on Health of People of Kishanganj, Bihar, India. International Research Journal of Environmental Sciences *4(5)*,46-56.
- Labuda, J., K Bubnicova, K., Kovalova, L., Vanickova, M., Mattusch, J., and Wennrich, R. (2005). Voltammetric detection of damage to DNA by arsenic compounds at a DNA biosensor. Sensors *5*, 411-423.
- Landis-Piwowar, K.R., and Iyer, N.R. (2014). Cancer chemoprevention: current state of the art. Cancer growth and metastasis *7*, 19.
- Lindberg, A.L., Rahman, M., Persson, L.A., and Vahter, M. (2008). The risk of arsenic induced skin lesions in Bangladeshi men and women is affected by arsenic metabolism and the age at first exposure. Toxicology and applied pharmacology *230*, 9-16.
- Lindgren, A., Vahter, M., and Dencker, L. (1982). Autoradiographic Studies on the Distribution of Arsenic in Mice and Hamsters Administered 74As-Arsenite or -Arsenate. Acta Pharmacologica et Toxicologica *51*, 253-265.
- Lindsey Torre, M.S.P.H., epidemiologist, American Cancer Society; David Katz, M.D., M.P.H., director, Yale University Prevention Research Center, New Haven, Conn., and president, American College of Lifestyle Medicine, (2015) Cancer Epidemiology, Biomarkers & Prevention.
- Liu, J., and Waalkes, M.P. (2008). Liver is a Target of Arsenic Carcinogenesis. Toxicological Sciences *105*, 24-32.
- Liu, Z. (2010). Roles of vertebrate aquaglyceroporins in arsenic transport and detoxification. Advances in experimental medicine and biology *679*, 71-81.
- Lu, G., Xu, H., Chang, D., Wu, Z., Yao, X., Zhang, S., Li, Z., Bai, J., Cai, Q., and Zhang, W. (2014). Arsenic exposure is associated with DNA hypermethylation of

the tumor suppressor gene p16. Journal of Occupational Medicine and Toxicology *9*, 1.

- Lynn, S., Gurr, J.R., Lai, H.T., and Jan, K.Y. (2000). NADH oxidase activation is involved in arsenite-induced oxidative DNA damage in human vascular smooth muscle cells. Circulation Research *86*, 514-519.
- Maciaszczyk-Dziubinska, E., Wawrzycka, D., and Wysocki, R. (2012). Arsenic and antimony transporters in eukaryotes. International journal of molecular sciences *13*, 3527-3548.
- Mandal, B.K., and Suzuki, K.T. (2002). Arsenic round the world: a review. Talanta *58*, 201-235.
- Mappes, R. (1977). [Experiments on excretion of arsenic in urine (author's transl)]. International archives of occupational and environmental health *40*, 267-272.
- Marafante, E., Vahter, M., and Envall, J. (1985). The role of the methylation in the detoxication of arsenate in the rabbit. Chemico-biological interactions *56*, 225-238.
- Martinez, V.D., Vucic, E.A., Becker-Santos, D.D., Gil, L., and Lam, W.L. (2011). Arsenic Exposure and the Induction of Human Cancers. Journal of Toxicology *2011*, 431287.
- Mateen, S., Raina, K., and Agarwal, R. (2013). Chemopreventive and anti-cancer efficacy of silibinin against growth and progression of lung cancer. Nutrition and cancer *65*, 3-11.
- Mittal, A., Elmets, C.A., and Katiyar, S.K. (2003). Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation. Carcinogenesis *24*, 1379-1388.
- Muenyi, C.S., Ljungman, M., and States, J.C. (2015). Arsenic Disruption of DNA Damage Responses-Potential Role in Carcinogenesis and Chemotherapy. Biomolecules *5*, 2184-2193.
- Ning, R.Y. (2002). Arsenic removal by reverse osmosis. Desalination *143*, 237-241.
- Pal, H.C., Sharma, S., Elmets, C.A., Athar, M., and Afaq, F. (2013). Fisetin inhibits growth, induces G2/M arrest and apoptosis of human epidermoid carcinoma A431 cells: role of mitochondrial membrane potential disruption and consequent caspases activation. Experimental dermatology *22*, 470-475.
- Pal, H.C., Sharma, S., Strickland, L.R., Katiyar, S.K., Ballestas, M.E., Athar, M., Elmets, C.A., and Afaq, F. (2014). Fisetin inhibits human melanoma cell invasion

through promotion of mesenchymal to epithelial transition and by targeting MAPK and NFκB signaling pathways. PloS one *9*, e86338.

- Panda, S. (2010). Nonmelanoma skin cancer in India: Current scenario. Indian journal of dermatology *55*, 373.
- Pelicano, H., Feng, L., Zhou, Y., Carew, J.S., Hileman, E.O., Plunkett, W., Keating, M.J., and Huang, P. (2003). Inhibition of mitochondrial respiration: a novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. The Journal of biological chemistry *278*, 37832-37839.
- Pilsner, J.R., Liu, X., Ahsan, H., Ilievski, V., Slavkovich, V., Levy, D., Factor-Litvak, P., Graziano, J.H., and Gamble, M.V. (2009). Folate deficiency, hyperhomocysteinemia, low urinary creatinine, and hypomethylation of leukocyte DNA are risk factors for arsenic-induced skin lesions. Environ Health Perspect *117*, 254-260.
- Pomroy, C., Charbonneau, S.M., McCullough, R.S., and Tam, G.K. (1980). Human retention studies with 74As. Toxicology and applied pharmacology *53*, 550-556.
- Porter, A.C., Fanger, G.R., and Vaillancourt, R.R. (1999). Signal transduction pathways regulated by arsenate and arsenite. Oncogene *18*, 7794-7802.
- Pourahmad, J., Rabiei, M., Jokar, F., and O'Brien, P.J. (2005). A comparison of hepatocyte cytotoxic mechanisms for chromate and arsenite. Toxicology *206*, 449-460.
- Rahman, M.S., Hall, L.L., and Hughes, M.F. (1994). In vitro percutaneous absorption of sodium arsenate in B6C3F(1) mice. Toxicology in vitro *8*, 441-448.
- Ralph, S.J. (2008). Arsenic-Based Antineoplastic Drugs and Their Mechanisms of Action. Metal-Based Drugs *2008*, 260146.
- Ren, X., Aleshin, M., Jo, W.J., Dills, R., Kalman, D.A., Vulpe, C.D., Smith, M.T., and Zhang, L. (2011a). Involvement of N-6 adenine-specific DNA methyltransferase 1 (N6AMT1) in arsenic biomethylation and its role in arsenic-induced toxicity. Environ Health Perspect *119*, 771-777.
- Ren, X., McHale, C.M., Skibola, C.F., Smith, A.H., Smith, M.T., and Zhang, L. (2011b). An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. Environ Health Perspect *119*, 11-19.
- Rosen, B.P. (2002). Biochemistry of arsenic detoxification. FEBS letters *529*, 86-92.

- Saha, J.C., Dikshit, A.K., Bandyopadhyay, M., and Saha, K.C. (1999). A Review of Arsenic Poisoning and its Effects on Human Health. Critical Reviews in Environmental Science and Technology *29*, 281-313.
- Saladi, R.N., and Persaud, A.N. (2005). The causes of skin cancer: a comprehensive review. Drugs of Today *41*, 37-54.
- Sengupta, S.R., Das, N.K., and Datta, P.K. (2008). Pathogenesis, clinical features and pathology of chronic arsenicosis. Indian journal of dermatology, venereology and leprology *74*, 559-570.
- Shankar, S., Shanker, U., and Shikha (2014). Arsenic Contamination of Groundwater: A Review of Sources, Prevalence, Health Risks, and Strategies for Mitigation. The Scientific World Journal *2014*, 18.
- Shen, S., Li, X.F., Cullen, W.R., Weinfeld, M., and Le, X.C. (2013). Arsenic Binding to Proteins. Chemical Reviews *113*, 7769-7792.
- Shi, H., Shi, X., and Liu, K.J. (2004). Oxidative mechanism of arsenic toxicity and carcinogenesis. Molecular and cellular biochemistry *255*, 67-78.
- Singh, M., Suman, S., and Shukla, Y. (2014). New enlightenment of skin cancer chemoprevention through phytochemicals: In vitro and in vivo studies and the underlying mechanisms. BioMed research international *2014*, 243452.
- Singh, R.P., and Agarwal, R. (2006). Prostate cancer chemoprevention by silibinin: bench to bedside. Molecular carcinogenesis *45*, 436-442.
- Smith, A.H., and Smith, M.M. (2004). Arsenic drinking water regulations in developing countries with extensive exposure. Toxicology *198*, 39-44.
- Steward, W., and Brown, K. (2013). Cancer chemoprevention: a rapidly evolving field. British journal of cancer *109*, 1-7.
- Straub, A.C., Stolz, D.B., Vin, H., Ross, M.A., Soucy, N.V., Klei, L.R., and Barchowsky, A. (2007). Low level arsenic promotes progressive inflammatory angiogenesis and liver blood vessel remodeling in mice. Toxicology and applied pharmacology *222*, 327-336.
- Sudhakar, A. (2009). History of Cancer, Ancient and Modern Treatment Methods. Journal of cancer science & therapy *1*, 1-4.
- Suh, Y., Afaq, F., Johnson, J.J., and Mukhtar, H. (2009). A plant flavonoid fisetin induces apoptosis in colon cancer cells by inhibition of COX2 and Wnt/EGFR/NF-κB-signaling pathways. Carcinogenesis *30*, 300-307.

- Sun, H.-J., Rathinasabapathi, B., Wu, B., Luo, J., Pu, L.-P., and Ma, L.Q. (2014). Arsenic and selenium toxicity and their interactive effects in humans. Environment International *69*, 148-158.
- Tallman, M., Lo-Coco, F., Barnes, G., Kruse, M., Wildner, R., Martin, M., Mueller, U., and Tang, B. (2015). Cost-effectiveness analysis of treating acute promyelocytic leukemia patients with arsenic trioxide and retinoic acid in the United States. Clinical Lymphoma Myeloma and Leukemia *15*, 771-777.
- Tapio, S., and Grosche, B. (2006). Arsenic in the aetiology of cancer. Mutation research *612*, 215-246.
- Tchounwou, P.B., Patlolla, A.K., and Centeno, J.A. (2003). Carcinogenic and systemic health effects associated with arsenic exposure--a critical review. Toxicologic pathology *31*, 575-588.
- Tokar, E.J., Benbrahim-Tallaa, L., Ward, J.M., Lunn, R., Sams, R.L., and Waalkes, M.P. (2010). Cancer in experimental animals exposed to arsenic and arsenic compounds. Critical reviews in toxicology *40*, 912-927.
- Tseng, C.H., Chong, C.K., Tseng, C.P., and Centeno, J.A. (2007). Blackfoot Disease in Taiwan: Its Link with Inorganic Arsenic Exposure from Drinking Water. Ambio *36*, 82-84.
- Tyagi, A.K., Singh, R.P., Agarwal, C., Chan, D.C., and Agarwal, R. (2002). Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth Inhibition, G2-M arrest, and apoptosis. Clinical Cancer Research *8*, 3512-3519.
- Tyler, C.R., and Allan, A.M. (2014). The Effects of Arsenic Exposure on Neurological and Cognitive Dysfunction in Human and Rodent Studies: A Review. Current Environmental Health Reports *1*, 132-147.
- Vahter, M. (1999). Methylation of inorganic arsenic in different mammalian species and population groups. Science progress *82 (Pt 1)*, 69-88.
- Vahter, M. (2009). Effects of arsenic on maternal and fetal health. Annual review of nutrition *29*, 381-399.
- Vahter, M., Concha, G., Nermell, B., Nilsson, R., Dulout, F., and Natarajan, A.T. (1995). A unique metabolism of inorganic arsenic in native Andean women. European journal of pharmacology *293*, 455-462.
- Vahter, M., and Norin, H. (1980). Metabolism of 74As-labeled trivalent and pentavalent inorganic arsenic in mice. Environmental research *21*, 446-457.

- Villa-Bellosta, R., and Sorribas, V. (2010). Arsenate transport by sodium/phosphate cotransporter type IIb. Toxicology and applied pharmacology *247*, 36-40.
- Wang, J.P., Qi, L., Moore, M.R., and Ng, J.C. (2002). A review of animal models for the study of arsenic carcinogenesis. Toxicology letters *133*, 17-31.
- Wang, Z.Y., Agarwal, R., Bickers, D.R., and Mukhtar, H. (1991). Protection against ultraviolet B radiation-induced photocarcinogenesis in hairless mice by green tea polyphenols. Carcinogenesis *12*, 1527-1530.
- Watanabe, T., and Hirano, S. (2013). Metabolism of arsenic and its toxicological relevance. Archives of toxicology *87*, 969-979.
- Webb, D.R., Sipes, I.G., and Carter, D.E. (1984). In vitro solubility and in vivo toxicity of gallium arsenide. Toxicology and applied pharmacology *76*, 96-104.
- Wollina, U. (2016). Arsenic and skin cancer–Case report with chemoprevention. Our Dermatology Online/Nasza Dermatologia Online *7(2)*,172-175.
- Yamamura S. (2001). Drinking water guidelines and standards. In United Nations Synthesis Report on Arsenic in Drinking Water (draft report), WHO, Geneva.
- Yang, H.-C., Fu, H.-L., Lin, Y.-F., and Rosen, B.P. (2012). Pathways of arsenic uptake and efflux. Current topics in membranes *69*, 325.
- Yedjou, C., Tchounwou, P., Jenkins, J., and McMurray, R. (2010). Basic mechanisms of arsenic trioxide (ATO)-induced apoptosis in human leukemia (HL-60) cells. Journal of hematology & oncology *3*, 28.
- Zhang, T.D., Chen, G.Q., Wang, Z.G., Wang, Z.Y., Chen, S.J., and Chen, Z. (2001). Arsenic trioxide, a therapeutic agent for APL. Oncogene *20*, 7146-7153.
- Zhang, X., Cornelis, R., De Kimpe, J., Mees, L., and Lameire, N. (1997). Speciation of arsenic in serum, urine, and dialysate of patients on continuous ambulatory peritoneal dialysis. Clinical chemistry *43*, 406-408.

Appendix

Appendix

A. Stock solutions of drugs/agents and dyes

Drug/agent	Stock concentration	Working concentartion
Arsenic	20 mM	0.05 µM - 3 µM
Silibinin	100 mM	100 µM
Fisetin	100 mM	25 µM
Propidium Iodide (PI)	5 mg/ml	25 µg/ml
Acridine orange (AO)	100 mg/ml	100 µg/ml
Ethidium Bromide (Et Br)	100 mg/ml	100 µg/ml

B. 10X PBS (1 Liter)

Dissolved the following constituents in 800 ml double distilled water

- NaCl: 80g
- KCl: 2.0g
- Na_2HPO_4: 14.4g
- KH_2PO_4: 2.4g

Adjusted pH to 7.4. Made up the volume up to 1 liter with double distilled water. Sterilized by autoclaving at 15 psi for 20 minutes. Stored at room temperature.

C. Crystal violet stain for colony formation assay

5 mg/ml crystal violet in 2% ethanol. Stored at room temperature.

D. Fixing solution for colony formation assay

12.5% acetic acid and 30% methanol in double distilled water. Stored at room temperature.

E. 2x Zymography sample buffer

Reagents required	Volume
0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol	2.0 ml
10% (w/v) SDS	4.0 ml
10% SD 0.1% Bromophenol Blue in DDH2O (w/v)	0.03 ml
Volume made up to 10 ml with double distilled water	

F. 10x Zymography renaturation buffer (100ml)

Reagents required	**Volume**
Triton X-100	25.0 ml
DD water to make	75.0 ml
Total	**100 ml**

G. 1x Zymography developing buffer (1 L)

Components	**Conc.**
Tris–HCl (pH 7.8)	50 mM
NaCl	200 mM
$ZnCl_2$	0.7 mg
$CaCl_2$	5 mM
Sodium azide	0.2 g
Brij 35	0.2% (v/v)
Adjusted to pH 7.5 with HCl and adjusted volume to 1000 ml with water.	

H. Coomassie R-250 staining solution

Components	**Conc./Volume**
Coomassie R-250	0.25 g
Methanol	40%
Acetic acid	7.5%
Volume made upto 1 L with double distilled water	

I. Coomassie R-250 destaining solution

Components	**Conc./Volume**
Methanol	5%
Acetic acid	7.5%
Volume made upto 1 L with double distilled water	

J. MTT Reagent preparation

5 mg/ml MTT was freshly prepared in autoclaved 1X PBS in sterile condition in laminar hood.

K. 0.5 x TBE electrophoresis buffer

Reagents required	**Amount/Volume**
Tris base	54 g
Boric Acid	27.5 g
0.5 M EDTA, pH 8.0	20 ml
EtBr	2.5 mg
DD water	**To make 1000 ml**

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