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CHEMOTHERAPEUTIC EFFICACY OF *WITHAFERIN-A* IN EXPERIMENTAL HEPATOCELLULAR CARCINOMA

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ABSTRACT

Hepatocellular Carcinoma is the leading cause of death among men worldwide. Chemoprevention and chemotherapy play beneficial roles in reducing the incidence and mortality of cancer. Cancer cell metabolism is characterized by an enhanced uptake and utilization of glucose, a phenomenon known as the Warburg effect. The persistent activation of altered metabolic enzymes is considered to be fundamental to the transformation of normal cells to cancer cells. Accordingly, interrupting cancer promoting metabolic sites may provide a promising strategy to halt tumor development. Identifying the abnormalities of cellular energy metabolism facilitates early detection and management of liver cancer. In view above, we tried to demonstrate dysregulated and reprogrammed cancer metabolism followed by clinical relevance of the metabolic enzymes, such as Glycolytic enzymes, gluconeogenesis, TCA cycle enzymes and glycoproteins. The present study was evaluated to check the effect of *withaferin-A* on cellular metabolic energy fluxes in DEN induced proliferative liver cancer. The results showed that the activities of glycolytic enzymes significantly increased and gluconeogenesis, tricarboxylic acid (TCA) cycle enzymes activities decreased in DEN induced liver cancer bearing rats. These altered metabolic enzymes were significantly normalized in the *withaferin-A* treated group rats. Our data suggest that *withaferin-A*, may extend its chemotherapeutic effect

through modulating the metabolic enzymes and glycoproteins, indicators of promising chemotherapeutic agent for cancer treatment.

KEYWORDS: *Withaferin-A*, glycolysis, gluconeogenic enzymes, TCA cycle, liver cancer, DEN.

1. INTRODUCTION

Metabolic reprogramming, now considered a hallmark of cancer, has become a major research area in cancer biology in the past decade.[1,2] The links between cancer and metabolism are multifaceted, which altered cancer cell metabolism resulting in modulation of intracellular signaling pathways that are interrupted by mutated oncogenes and tumor suppressor genes. The characteristic metabolic hallmark of tumor metabolism is aerobic glycolysis.[3] In this regard, mutant metabolic enzymes can drive tumorigenesis and conversely, cancer genes regulate metabolism, which means alterations in cell metabolism may trigger tumorigenesis. Cancer cells have distinct metabolism and highly depend on glycolysis, gluconeogenesis instead of mitochondrial oxidative phosphorylation. The correlation between glycolytic ATP production and tumor malignancy has been reported.[4] It was originally hypothesized that these metabolic changes reflect damage to mitochondrial oxidative phosphorylation, implying that cancer cells could not respire properly to obtain sufficient ATP.

However, recent studies revealed that many cancer cells are capable of synthesizing ATP through mitochondrial respiration.[5] Regardless of whether mitochondrial respiration is reduced, the fact remains that cancer cells exhibit high rates of glycolysis and lactate fermentation, and this dependence on glucose utilization may be exploited for therapeutic intervention. During the past decade, many studies focusing on mitochondrial function in cancer cells have suggested that the Warburg effect is more closely related to alterations in signaling pathways that control the uptake and utilization of glucose than to mitochondrial defects.[6] The Warburg effect has recently gained attention again in the cancer research field, because researchers have begun to re-evaluate the significance of aerobic glycolysis in tumor cells.[7] So understanding the complex cancer energy metabolism will help to develop new approaches in early diagnosis and treatment of cancers.

Since targeting metabolism is a new strategy to develop a new class of anticancer drugs, alike, the Warburg effect which describes the altered cancer metabolism, alterations of

metabolic genes that could provide a direct genetic link to altered metabolism.[8] Cellular metabolic enzyme is regulated by many oncogenes and also serves a dual function as the speed regulator of cancer cell metabolism.[9] For example, the expression and translation of hexokinase, phosphoglucoisomerase, aldolase and pyruvate kinase are controlled by many oncogenes and metabolic intermediates.[10] May cancer cells acquire the rapid growth phenotype and ultimately develop hepatocellular carcinoma through energy producing molecules. This report is focusing on the analysis of metabolic enzymes activities that are involved in tumor development and discuss the current strategies of targeting metabolic pathways for cancer treatment. Herbal treatment for cancer has almost no side effects and is relatively cheap and locally available. They are effective in reducing the tumor growth in the system.[11] So the purpose of this study is to demonstrate that modulatory effect of *withaferin-A* by on glycolysis, gluconeogenesis enzymes, TCA cycle enzymes and glycoproteins in liver cancer induced by DEN. There is no information available on *withaferin-A* in liver cancer interactions with reference to metabolic enzymes levels. The objective of the present study is to emphasize the modulation of the selective metabolic enzymes by *withaferin-A* in chemically induced hepatocellular carcinoma.

2. MATERIALS AND METHOD

2.1. Animals: Healthy Male Wistar albino rats' weighing130-150kg was used in this work. The animals were purchased from Central Animal House Facility, Dr. ALM PGIBMS, University of Madras, Taramani, Chennai-600 113, India, and maintained in a controlled environmental condition of temperature (23 ± 2°C) and relative humidity (50-70%) on alternatively 12 hr light/dark cycles. All animals were fed standard pellet diet (Gold Mohor rat feed) and water ad libitum. The rats were sanctioned and approved by the Institutional Animal Ethical Committee (IAEC NO. 02/016/08).

2.2. Experimental design: The experimental animals were divided into five groups with six rats each. Group I- Control animals treated with 0.5% carboxymethyl cellulose (CMC) orally as vehicle throughout the experimental period. Group II- Hepatocellular carcinoma induced animals by providing 0.01% diethylnitrosoamine (DEN) through drinking water for 15 weeks. Group III- Animals simultaneously treated with DEN (as in Group II) on day one and then treated with *Withaferin-A* (50 mg/kg b.wt) for 21 days. Group IV- Hepatocellular carcinoma bearing animals post treated with *Withaferin-A* (as in Group III) after the confirmation of cancer. Group V- Control animals treated with *Withaferin-A* (as in Group III) alone for 21

days. After 18 weeks of experimental period, the animals were sacrificed by cervical decapitation. The blood serum and liver tissue was subjected to following biochemical parameters analysis.

2.3. Biochemical assays: The mitochondria were isolated by the method of Johnson and Lardy.[12] The enzyme Isocitrate dehydrogenase activity was assayed according to the method of King[13], the activity of alpha-ketoglutarate dehydrogenase was assayed by the method of Reed and Mukkerjee[14], the activity of Succinate dehydrogenase was assayed according to the method of Slater and Bonner[15], the enzyme activity of Malate dehydrogenase was assayed by the method of Mehler et al.[16] Hexokinase activity was assayed by the method of Brandstrup et al.[17] Phosphoglucoisomerase was assayed according to Horrocks et al.[18] Aldolase was estimated by the method of King.[13] The activities of glucose-6-phosphatase and fructose-1,6-disphosphatase were assayed by the method of Gancedo and Gancedo.[19] Protein was estimated by the method of Lowry et al.[20] Plasma glucose, albumin was estimated using commercial kit. The hexose level was estimated using the method of Niebes.[21] Hexosamine content was estimated using the method of Wagner.[22] The sialic acid level was determined using the method of Warren.[23]

2.4. Statistical analysis: For statistical analysis, one-way analysis of variance (ANOVA) was used, the levels of significance were evaluated with p-values.

3. RESULTS

3.1. Glucose and proteins level: Fig. 1 & 2. displays the effect of *withaferin-A* on the activities of glucose and protein levels in the plasma, serum of control and experimental animals. The activities of the glucose, proteins were found to be significantly ($p<0.001$) decreased in cancer induced group II animals when compared with the control animals (G-I). *Withaferin-A* administration groups caused a significant increase in the activities of the glucose, proteins levels when compared with cancer bearing group. However, there found to be no significant difference in the activities of the glucose, proteins between the control animals and the control animals treated with *withaferin-A*.

3.2. Glycolytic enzyme activities: Fig.3. shows the activities of glycolytic enzymes in liver tissues of rats. The activities of hexokinase, phosphoglucoisomerase and aldolase levels significantly increased ($P<0.05$ vs. control) in DEN-induced liver cancer bearing rats. However, *withaferin-A* treatment for 21 days before the administration of DEN in Group III

rats and post-treatment with *withaferin-A* in liver cancer bearing Group IV rats significantly reduced the activities of hexokinase, phosphoglucoisomerase, and aldolase compared with tumor bearing rats (Group II). Group V showed no effective changes (P<0.05) when compared with the control rats (Group I).

3.3. Gluconeogenic enzymes: Fig. 4. shows the gluconeogenic activities in the liver tissue of DEN induced liver cancer along with *withaferin-A* pre-and post-treated rats. The activities of gluconeogenic enzymes such as fructose-1,6-bisphosphatase and glucose-6-phosphatase significantly decreased (P< 0.05) in DEN induced liver cancer bearing rats when compared with the control rats (Group I). However, *withaferin-A* pre-treated rats showed a significant increase of fructose-1,6-bisphosphatase and glucose-6-phosphatase activities (P<0.05 vs. induced). Furthermore, post-treatment with *withaferin-A* (50 mg/kg body weight) for 21 days significantly restored the activities of fructose-1,6-bisphosphatase and glucose-6-phosphatase in Group IV compared with cancer bearing rats (P<0.05). No significant enzyme activities were found in rats treated with *withaferin-A* alone (P<0.05 vs. control).

3.4. Mitochondrial Enzymes: Table 1. displays the effect of *withaferin-A* on the activities of mitochondrial enzymes such as alpha-KGDH, IDH, SDH and MDH in the liver of control and experimental animals. The activities of the enzymes were found to be significantly (p<0.001) decreased in cancer induced group II animals when compared with the control animals (G-I). *Withaferin-A* (G-III) administration caused a significant increase in the activities of the mitochondrial enzymes when compared with cancer bearing group. Cancer bearing animals treated with *withaferin-A* (G-IV) showed a much significant (p<0.001) increase in these enzyme activities when compared with the cancer bearing animals. However, there found to be no significant difference in the activities of the mitochondrial enzymes between the control animals and the control animals treated with *withaferin-A*.

3.5. Glycoproteins: Table 2 & 3. shows the effects of *withaferin-A* on the levels of glycoproteins in the plasma and liver of control and experimental animals. The levels of all three glycoproteins (hexose, hexosamine and sialic acid) were found to be significantly (P<0.001) increased in cancer bearing animals (group II) compared with the control group (group I). Treatment with *withaferin-A* (group IV) caused a significant (P<0.001) decrease in their levels compared with the cancer-induced group (group II). When comparisons were made between the *withaferin-A* pre-treated (group III) and post-treated (group IV) animals, a significant (P<0.001; P<0.01) decrease in the levels of hexosamine and sialic acid was

observed. There was no significant difference in the glycoprotein levels between the control animals (group I) and the control animals treated with *withaferin-A* alone (group V).

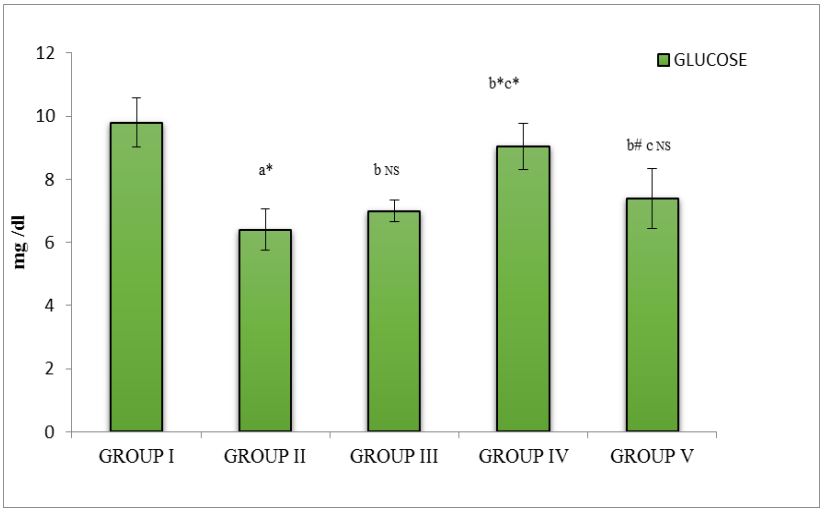

Fig.1. The level of blood glucose in control and experimental rats

Values are expressed as mean ± SD for six rats in each group

a - as compared with group I; b - as compared with group II;

c - as compared with group III

Statistical significance: *p<0.001; @p<0.01; #p<0.05

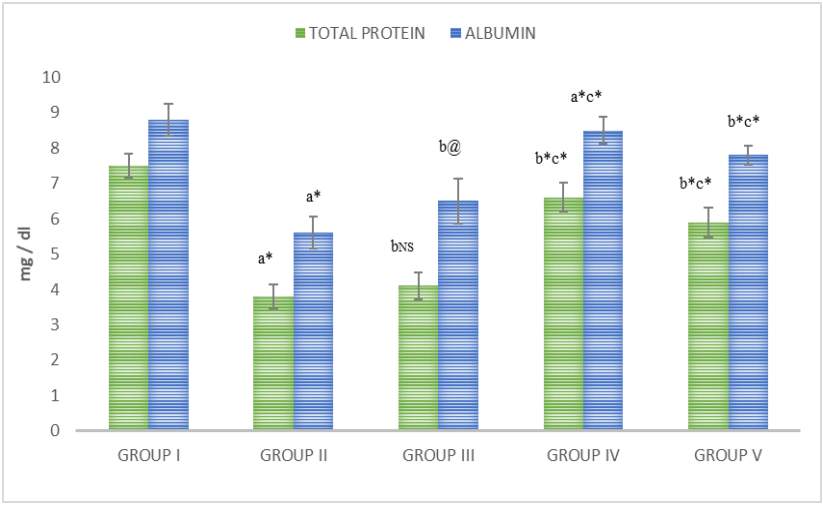

Fig.2. The levels of total protein and albumin in serum of control and experimental rats

Values are expressed as mean ± SD for six rats in each group

a - as compared with group I; b - as compared with group II;

c - as compared with group III

Statistical significance: *p<0.001; @p<0.01; #p<0.05

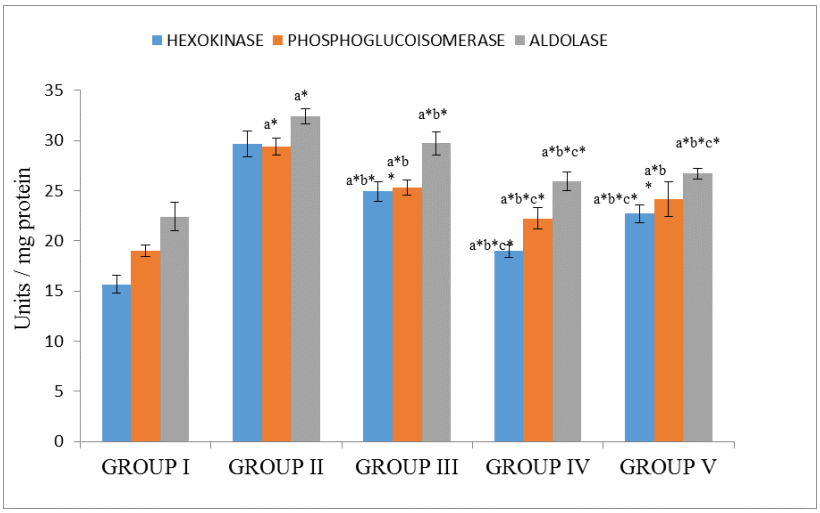

Fig.3. Effect of *withaferin-A* on glycolytic enzymes in liver of control and experimental rats

Values are expressed as mean ± SD for six rats in each group

Hexokinase - nmoles of glucose-6 phosphate formed / min / mg protein

Phosphoglucoisomerase - nmoles of fructose formed / min / mg protein

Aldolase - nmoles of glyceraldehyde formed / min / mg protein

a - as compared with group I; b - as compared with group II;

c - as compared with group III, Statistical significance: *p<0.001; @p<0.01; #p<0.05

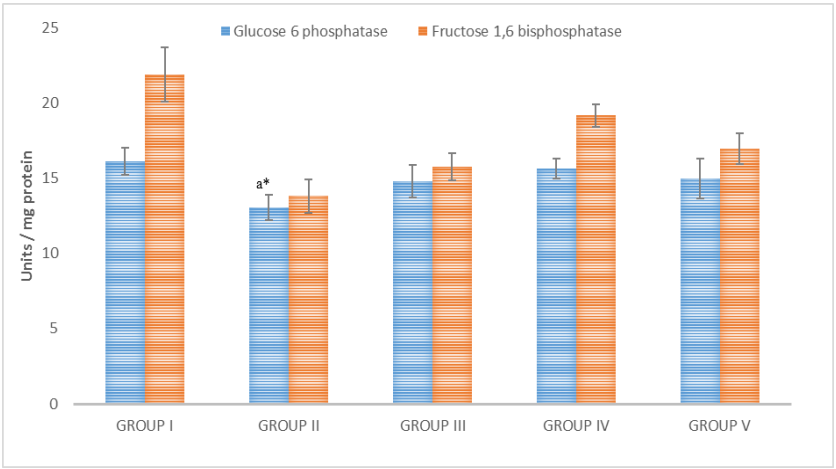

Fig.4. Effect of *withaferin-A* on gluconeogenic enzymes in liver of control and experimental rats

Values are expressed as mean ± SD for six rats in each group

Glucose 6 phosphatase & Fructose 1,6 bisphosphatase - nmoles of Pi released / min / mg protein a - as compared with group I; b - as compared with group II;

c - as compared with group III, Statistical significance: *p<0.001; @p<0.01; #p<0.05

Table 1. Effect of *Withaferin-A* on the activities of mitochondrial enzymes in liver of control and experimental animals

Particulars	Group I	Group II	Group III	Group IV	Group V
α-KG	0.54 ±0.05	0.32 ±0.03^{a*}	0.42 ±0.04^{b*}	0.46 ± 0.04$^{b*c@}$	0.55 ±0.05
ICDH	2.75 ±0.16	1.33 ± 0.12^{a*}	2.24 ± 0.14^{b*}	2.56 ± 0.12^{b*c*}	2.72 ± 0.17
SDH	4.84 ± 0.31	2.32 ± 0.25^{a*}	3.75 ±0.32^{b*}	3.86 ± 0.34$^{b*c@}$	4.75 ± 0.15
MDH	3.58 ± 0.23	1.75 ± 0.15^{a*}	2.74 ± 0.17^{b*}	3.67 ± 0.26$^{b*c\#}$	3.62 ± 0.57

Each value is expressed as mean ±SD for six mice in each group. ICDH- nmoles of α-KGDH liberated/min/mg protein. SDH- mmoles of succinate oxidised/min/mg protein. α-KGDH- mmoles of potassium ferrocyanide liberated/min/mg protein. MDH- nmoles of NADH oxidised/min/mg protein. a: Group II compared with Group I; b: Group II compared with Group III and IV. c- as compared with group III. Statistical significance: *p<0.001 @p<0.01 #p<0.05.

Table.2. Effect of *withaferin-A* on the levels of glycoproteins in plasma of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV	Group V
Hexose	11.82 ± 1.12	22.22 ± 1.81$^{a\#}$	18.10 ± 1.63$^{b\#}$	14.79 ± 1.23$^{b\#}$	11.85 ± 1.11$^{b\#}$
Hexosamine	14.52 ± 1.31	21.74 ± 1.84$^{a\#}$	18.74 ± 1.68$^{b\#}$	17.05 ± 1.43$^{b\#}$	14.45 ± 1.28$^{b\#}$
Sialic acid	20.87 ± 1.76	12.54 ± 1.84$^{a\#}$	15.16 ± 1.25$^{b\#}$	17.35 ± 1.53$^{b\#}$	21.24 ± 1.68$^{b\#}$

Each value is expressed as mean ± SD for six rats in each group.

Units: mg/dl. Comparisons are made between: 'a'- Group II Vs Group I; 'b'-Group II Vs Group III, IV and V. Significance: *p<0.001; @p<0.01; #p<0.05; NS - Not Significant.

Table.3. Effect of *withaferin-A* on the levels of glycoproteins in liver of control and experimental animals

Particulars	Group I	Group II	Group III	Group IV	Group V
Hexose	2.86± 0.24	3.74 ± 0.33$^{a\#}$	3.15 ± 0.25$^{b@}$	3.22 ± 0.23$^{b@}$	2.74 ± 0.23$^{b\#}$
Hexosamine	2.18 ± 0.23	3.80 ± 0.33$^{a\#}$	2.87 ± 0.24$^{b@}$	3.11 ± 0.26$^{b@}$	2.48 ± 0.20$^{b\#}$
Sialic Acid	1.65 ± 0.18	3.20 ± 0.27$^{a\#}$	2.86 ± 0.25$^{b\#}$	2.73 ± 0.31^{b*}	2.05 ± 0.21$^{b\#}$

Each value is expressed as mean ± SD for six rats in each group.

Units: mg/g of defatted tissue, a - as compared with Group I; b - as compared with Group II Statistical significance - $^{\#}$p<0.001, $^{@}$p<0.01, *p<0.05.

4. DISCUSSION

Cancer cell metabolism has recently become one of the most exciting and promising fields for the development of new anticancer agents. Cellular metabolic enzyme is regulated by many oncogenes and it serves a dual function as the speed regulator of cancer cell metabolism.[9] Metabolic targeting for cancer therapy is currently under investigation in an effort to identify small molecules that might specifically inhibit key metabolic enzyme steps associated with tumor growth. Altered metabolism is considered to be fundamental to the transformation of normal cells to cancer cells, and it is believed to be conserved in most tumours, including solid tumours, lymphoma and leukaemia.[24] Although the molecular mechanisms still remain largely unknown, mutations of oncogenes and tumour suppressors account for part of the metabolic reprogramming in cancer cells. In this regard, the understandings of new cancer metabolic profiles give us a hope that a new class of therapeutic agents may be developed for cancer therapy. Numerous analogs of metabolites are being tested at present as potential drug candidates to target tumour metabolism.[3,25,26] However, it remains unclear whether these benefits are similar and equally effective both in prevention and in treatment of cancer. Therefore, effective use of metabolic inhibitors may provide clinically favourable therapeutic strategy.

Cancer cells needs a vast amount of energy in a short period of time in order to proliferate. Glucose is the primary source of energy and is the main fuel for cellular respiration. Therefore, a high rate of glucose utilization is may occur in the cells to counterbalance of the increased energy needs involving in tumor progression. Specifically, glycolysis has long been considered the main source of energy for the cancer cell proliferate.[27] Cancer cells tend to produce ATP mainly by aerobic glycolysis, a metabolic shift characterized by high glucose uptake and increased production of lactate. Higher rate of glucose utilization and an increased production of lactic acid are characteristic features of neoplastic cells.[28] Previous report showed that glycolytic enzymes, strongly induced in the cancer cell may possess different biological functions, acting as both facilitators and gatekeepers of malignancy.[29,30] So the expression and translation of hexokinase, phosphoglucoisomerase, aldolase and pyruvate kinase are controlled by many oncogenes and metabolic intermediates.[10] From these above studies it showed that the cancer cells acquire the rapid growth phenotype and ultimately develop hepatocellular carcinoma through energy producing molecules. In this sense, attenuation or inhibition of glycolysis has been found useful for preventing the development of cancer, demonstrating that glycolysis is essential for proliferation, invasion and metastasis

of cancer.[31,32,33] Glycolysis can be blocked by inhibition of the glycolytic enzymes HK, PFK and pyruvate kinase (PK), all of which regulate irreversible and rate limiting steps in glycolysis.

Therefore, the strategy is to compromise or completely block the increased glycolysis in cancer cells by abating the enzymatic activity of these three proteins. Alterations in the activities of phosphoglucoisomerase might be expected by the influence of a proportion of glucose 6-phosphate, which is metabolized via the glycolytic pathway.[34] Upon *withaferin-A* treatment, liver cancer cells could decrease glycolytic regulatory enzyme activities, which inhibits accelerated glycolytic activities and induces the apoptosis in malignant tumor cells. The diminished activities of fructose-1,6-bisphosphatase and glucose-6-phosphatase were due to higher concentration of lactic acid production in neoplastic tissues.[35] Gluconeogenesis may play an important role in essential energy- requiring process of the host; thus, contributing to promote weight loss.[36] Therefore, targeting glycolysis/gluconeogenesis pathways as a consequence of metabolic reprogramming is an attractive therapeutic strategy because it is central to the growth and survival of cancer cells.[37] Similarly, we observed decreased level of gluconeogenesis enzymes in cancer bearing rats. However, the levels of these gluconeogenesis enzymes levels were near-normal in the *withaferin-A* treated animal. This is due to the anticancer properties of *withaferin-A*.

Alterations in mitochondrial functions i.e., maintenance of ion homeostasis or ATP supply, have repeatedly been suggested to contribute to cellular transformation. Tumour cell mitochondria can differ structurally and functionally from normal cells, but clear evidence in favours of this suggestion is lacking. However, some chemical carcinogen primarily attacks mitochondria which is the molecular clocks in eukaryotes.[38] ICDH refers to the NADP+ dependent enzyme, which present in cytoplasm and mitochondria in several tissues. SDH is a marker enzyme in TCA cycle and succinate, phosphate, ATP promote its activity. Being a regulatory enzyme its property is altered when it is solubilised. Availability of oxalate is controlled by another chief enzyme in TCA cycle is MDH, which converts malate to oxaloacetate. In the present study we have observed decreased activities of TCA cycle key enzymes such as ICDH, SDH, MDH and alpha-KGDH in liver cancer bearing animals. This kind of decrease in mitochondria enzymes might be due to the marked indicator of metabolic changes, reduced number of mitochondria, deficiency in one or more electron transport chain components in cancer cells.[39] Novel therapies showed that the use of plant metabolites, for

treatment of liver cancer by inducing apoptosis in the cancer cells by many cellular mechanisms.[40] Begum and Sadique[41] have reported that *Withaniasomnifera* influenced the level of SDH in air pouch granuloma. *Withaferin-A* treated group shows a significant increase in the activities of ICDH, SDH, MDH and alpha-KGDH when compared with group II animals. The result of the present study strongly indicates that the *withaferin-A* has the antioxidant and anticancer property and promises therapeutic agent. This result also holds potential relevance in terms of cancer chemotherapy in experimental liver cancer.

Glycoproteins are common components of animal cell surfaces and are constituents of lysosomes, which are among the exported products by the cell. Carbohydrate moieties of glycoproteins have been implicated in the transport of metabolites across cell membranes, acting as mediators of immunological specificity and a direct relationship between glycoproteins and tumorigenesis has been observed.[42, 43] Similar results were observed in our experiment, the levels of hexose, hexosamine and sialic acid were increased in the liver cancer bearing group II animals. However, the level of these glycoproteins were near-normal in the *withaferin-A* treated animal, which may be the direct reaction of *withaferin-A* with free radicals, and the toxic reactive intermediates of DEN. This reduced level of glycoproteins components indicates that the drug has the ability to suppress malignancy by modulating cell transformation by controlling cell proliferation.

5. CONCLUSION

In conclusion, we were able to demonstrate the modulatory activity of *withaferin-A* on TCA cycle enzymes, electron transport chain complexes and other metabolic enzymes activity levels. This indicates that *withaferin-A* could conceivably protect against chemically induced carcinogenesis. From the observation it suggests that, pre- and post-treatment with *withaferin-A* in liver cancer could prevent abnormal carbohydrate metabolic energy fluxes and brought back to normal the functions of cells in energy metabolism. In the present study and the earlier reports confirm the antioxidant and anticancer activity of *withaferin-A* in the experimental cancers. Given the explosion of interest and information on cancer metabolism, it is hoped that new therapies will emerge from the basic sciences of metabolism in the next decade.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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