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DIRECT ACTIVATOR OF AMPK FROM SHILAJIT: A BIOINFORMATICS-BASED STUDY

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ABSTRACT: Metabolic syndrome is continuously on the rise, due to a changed lifestyle and long ageing. The abnormal function of mTOR/AMPK, a cellular energy switch, is the main cause behind this pathogenesis, which is now defined as "metabolic syndrome". Here, we have screened the metabolites of shilajit, obtained from the HRAMS analysis, to select the direct activators of AMPK, by using computational exploration, through molecular docking and ADMET prediction. The shilajit has been in clinical use in Ayurvedic medicine, for centuries, to enhance the overall vitality, immunocompetence and aphrodisiac potential, but scientific data are lacking to support its therapeutic claims. The shilajit, of Upakarma Ayurveda was purchased from the market and analyzed through HRAMS, which reported the presence of 5467 metabolites. Their CID numbers were obtained from the PubChem portaland docked against the AMPK (AMPK active site PDB ID-4CFF), by using LibDock and Discovery Studio to do a structure-based screening, ADME (absorption, distribution, metabolism, excretion) and toxicity prediction. Among them, Reproterol (CID-25654) and Ambruticin (CID-6918547) showed the best binding energy, in comparison to standard drug "A-769662 (CID: 54708532)". These metabolites can be used as a lead molecule to develop novel activators of AMPK. This study also supports the therapeutic claims of the use of Shilajit, for the management of metabolic syndrome. Helps in understanding its mechanism of action for other claims, involving AMPK-linked pathways.

INTRODUCTION: Obesity is the primary cause of several NCDs. Besides the control of excess energy intake as food, another approach to control obesity is to enhance the internal processes of energy expenditure. The AMP-activated protein kinase (AMPK) is one of the targets. It is naturally expressed during ATP depletion, either by food restriction by enhanced heavy physical activities or by uncoupling the mitochondrial phosphorylation.

Low AMPK activity has been implicated in diseases, like obesity, insulin resistance, type 2 Diabetes, cardiovascular diseases, Non-Alcoholic Fatty Liver Disease (NAFLD) and cancer involving abnormal energy homeostasis. The AMPK promotes glucose uptake in skeletal muscle and inhibits gluconeogenesis in the liver.

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Low AMPK activity in adipose tissue and skeletal muscle is also attributed to lipid accumulation and obesity. The dysregulation of AMPK in the hypothalamus may regulate appetite and energy expenditure, contributing to obesity. In Cardiovascular Diseases, low AMPK activity contributes to endothelial dysfunction, hypertension, myocardial hypertrophy, and increased susceptibility to myocardial infarction, so its activation is reported for vasodilation, inhibition of cardiac hypertrophy and protection against ischemia-reperfusion injury.

In the case of Non-Alcoholic Fatty Liver Disease (NAFLD), the low AMPK activity in the liver activates hepatic lipogenesis, whereas AMPK activation in the liver inhibits lipogenesis and promotes fatty acid oxidation. Low AMPK is also reported in neurodegenerative diseases by dysregulation of neuronal energy homeostasis, mitochondrial function and autophagy in neurons, resulting in Alzheimer's disease, Parkinson's disease, and Huntington's disease. In the case of Cancer, low AMPK activity is reported to promote tumour growth, metastasis and resistance to chemotherapy or radiotherapy. Its activation shows tumor-suppressive effects by inhibiting cell proliferation, promoting apoptosis and regulating cellular metabolism in cancer cells.

Thus, AMPK serves as a central hub to connect various pathways involved in NCDs, like the metabolism of glucose, through its increased cellular uptake and inhibition of gluconeogenesis; metabolism of lipids through promoting fatty acid oxidation and inhibiting lipogenesis, cell growth through suppression of mTOR signalling and promoting autophagy, managing oxidative stress and inflammation, activating antioxidant defence mechanisms and regulation of mitochondrial function [1-8].

Therefore, targeting AMPK activation may offer therapeutic opportunities for treating these diseases, either by enhancing the genetic expression of AMPK protein or by enhancing its catalytic turnover, which can be achieved by allosteric regulation. This may be indirect, by enhancing the cellular AMP level or by directly acting on the non-AMP allosteric site on the AMPK protein [9] have presented the crystal structure of human AMPK in complex with a small molecule activator that binds at a site between the kinase domain and the carbohydrate-binding module. Taking the lead from that, we have explored a library of metabolites, obtained from the HRAMS analysis of Shilajit and found 2 metabolites, showing better activity than the existing drug "A-769662" (6,7-dihydro-4-hydroxy-3-(2′-hydroxy[1,1′-biphenyl]-4-yl)-6-oxo-thieno [2, 3-b] pyridine-5-carbonitrile or 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), developed by Abbot [10].

The AMPK is a heterotrimeric protein kinase, phosphorylating at the serine/threonine residue of its target. It has three subunits (α, β, and γ), having tissue-specific different combinations. The AMP binds to the γ subunit, which induces conformational changes to expose it's α subunit, which is responsible for its kinase property. Its most important substrate is the tumour suppressor LKB1. There are indirect activators of AMPK, like Metformin and Berberine have been reported to inhibit the mitochondrial respiratory chain complex I. The direct activators, like AICAR, A-769662, Compound-13, PT-1 etc are also available which directly bind the AMPK protein to its other allosteric site. The metformin activates AMPK by inhibiting complex I of the mitochondrial electron transport chain contributing to a raised AMP:ATP ratio.

In the group of direct activators, the AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide) is reported to bind to the γ-subunit of the AMPK enzyme. It is taken up by cells and converted to ZMP (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5'-monophosphate), which is structurally similar to AMP and activates AMPK. The A-769662 is another synthetic direct AMPK activator, which binds to the β-subunit of the enzyme. Resveratrol, a natural product activates AMPK by directly binding to the γ-subunit of the enzyme, similar to AICAR [11-12].

Here, we have explored the potential of Shilajit, (asphaltum), towards its action on AMPK, which has been in clinical use for centuries to manage a variety of non-communicable diseases and to maintain overall wellness. The metabolites like phenolic, tannins, alkaloids, flavonoids, glycosides, and terpenoids, have been reported in shilajit [13], but

its standardization and quality control are still major challenges, due to the lack of marker metabolites. Currently, some metabolites like Dibenzo-alpha pyrones, urolithin A, urolithin B acetophenone *etc*, are being used for this purpose. Recently, we have identified some novel metabolites in Shilajit by using HRAMS, which may be used for standardization of Shilajit [14].

The genesis of shilajit is claimed to be the microbial-degraded product of bryophytes, in mountains, over a long period of several years. This is the reason, why the quality of shilajit depends on the geological location of the mountain rocks, from where it has been collected as raw material for extraction, purification and drug development. Its main therapeutic claims include it's antiaging and aphrodisiac, immunomodulator, skin diseases, wound healing, urogenital dysfunctions, antidiabetic, neurotropic, anti-arthritis, anti-hypertensive and heart diseases. It is also claimed to be an efficient drug vehicle, so it is used in combination with several other herbs. Some reports indicate the capability of shilajit to modulate the activity of testosterone in males [15-25].

METHODS: The shilajit of a branded company namely Upakarma Ayurveda, India was purchased online and subjected to its analysis by HRAMS, in the Central Discovery Centre (CDC) of Banaras Hindu University. Its analytical report showed the presence of 5467 metabolites. After data cleaning total of 1599 metabolites were selected for Lipdoc high throughput screening against AMPK active site (PDB ID: 4CFF) [26].

Among them, 163 metabolites failed to dock with AMPK and the remaining metabolites could bind with 2 subunits of AMPK, *i.e.* Alpha2 and Beta1. Among them, 1183 metabolites were screened, but their 2D visualization did not show accurate bonding AAs, so they were further subjected to 11 poses of grid-making to visualize the appropriate binding site-related data, which generated 51388 poses and among them, only 137698 conformers showed different configurations. Finally, 1183 metabolites indicated binding energy, higher than the standard compound, which is 64.65 in the docking, were opened in Venny software to remove the repetition and opened in the SwissADME portal. Only 1104 metabolites were found to be non-toxic and non-mutagenic. Out of them, 10 metabolites, having the highest binding energy, were chosen for further studies. Among them, only 7 metabolites had 3D SDF files, so they were processed with the CDOCKER portal for Docking optimization, by using the Grid size (X=-23.98, Y= -10.24, Z= 207.95, radios= 15.72, sphere). Based on the docking score, all 7 metabolites successfully docked have different binding energy and CDOCKER interaction energy. Further, these metabolites were subjected to a test for their drug-likeness, through "The Lipinski rule of 5".

RESULTS: The standard agonist of AMPK, A-769662 (CID: 54708532) was docked against AMPK active site PDB ID-4CFF, and its binding energy was found to be 64.653. Among them, 2 metabolites showed CDOCKER energy in negative, but the other 5 metabolites showed positive binding energy. The A-769662 showed a binding energy of -19.39 and a binding interaction of -49.57, but 6 metabolites of shilajit, had better binding scores **Table 1** and **2**. The 2D and 3D pictures of the receptor-ligand complex are given in **Fig. 1**. Though, all 7 metabolites could pass the criteria for drug-likeness on "Lipinski rule of 5" but only 2 metabolites showed the overall drug-likeness parameters of ADMET, which were Reproterol (CID-25654) and Ambruticin (CID-6918547).

So, they were docked against PDB ID-4CFF, and the 2D and 3D pictures of the receptor-ligand complex were made **Fig. 2** and **3**. The results of the interaction of Reproterol to the binding site showed the presence of van der Waals, conventional hydrogen bond, carbon-hydrogen bond, alkyl and pi-alkyl type of bonds with Amino acids (Lys^{31}, Leu^{18}, Val^{113}, Val^{81}, Ile^{46} and Phe^{90}). The results of the interaction of Ambruticin (6918547) at the binding site showed Asp^{88}, Val^{11}, Lys^{31}, Thr^{31}, Thr^{21}, Asp^{20}, His^{109}, Arg^{107} and Ile^{46} residues.

TABLE 1: LIST OF THE TOP 10 METABOLITES HAVING THE HIGHEST BINDING ENERGY, ALONG WITH THEIR PROPERTIES, AS PER HRAMS DATA

S. no.	Name of metabolites	CID	m/z	RT [min]	Area (Max.)	AUC x 10^7
1	Reproterol	25654	390.17563	12.771	48958507.58	4.90

2	Didodecyl-3,3-thiodipropionate (DLTDP)	31250	532.43945	26.002	19798707.96	1.98
3	Scutellarin	185617	463.08772	10.719	422257.6659	0.04
4	Lafutidine	5282136	430.18167	9.553	2412689.803	0.24
5	Ambruticin	6918547	455.28073	20.622	1297766.088	0.13
6	C14-Dihydroceramide	10255824	512.50366	26.877	2081695.404	0.21
7	Zizybeoside I	11972301	431.15674	13.309	1070934.835	0.11
8	16-feruloyloxypalmitic acid	14018343	466.31656	25.185	9896076.416	0.99
9	Pemetrexed	135410875	428.15515	10.77	7486140.179	0.75
10	Tetrahydrofolic acid	135444742	446.18045	17.202	2930708.626	0.29

TABLE 2: LIST OF 7 METABOLITES, WHICH PASSED THE LIPINSKI RULE OF 5 FOR DRUG-LIKENESS WITH THEIR BINDING ENERGY

S. no.	Name	CID	-Cdocker Energy	-Cdocker Interaction Energy
1	Reproterol	25654	49.2509	61.1519
2	Scutellarin	185617	21.5586	52.0744
3	Lafutidine	5282136	30.7794	56.4332
4	Ambruticin	6918547	-33.2458	62.7439/64.653
5	Zizybeoside I	11972301	-6.00685	47.7803
6	Pemetrexed	135410875	55.261	61.1214
7	Tetrahydrofolic acid	135444742	54.5865	55.1234
8	A 769662 (Standard)	54708532	19.39	49.57

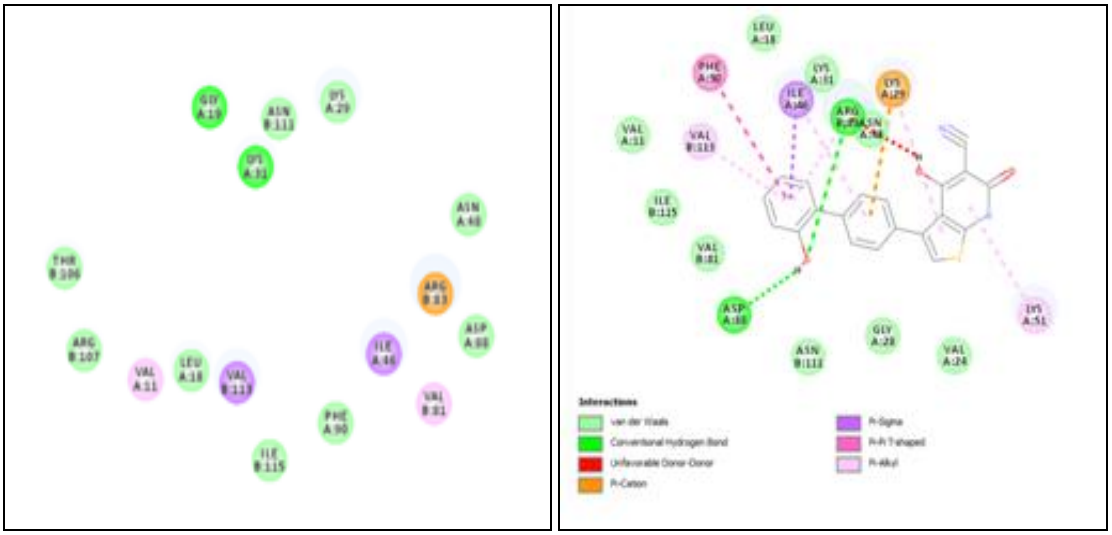

FIG. 1A: 2D PICTURE OF THE EMPTY RECEPTOR OF AMPK (PDB ID: 4CFF) AND FIG. 1B: SHOWING A 2D PICTURE OF THE STANDARD DRUG (A-769662), COMPLEXED WITH RECEPTOR AMPK (PDB ID: 4CFF)

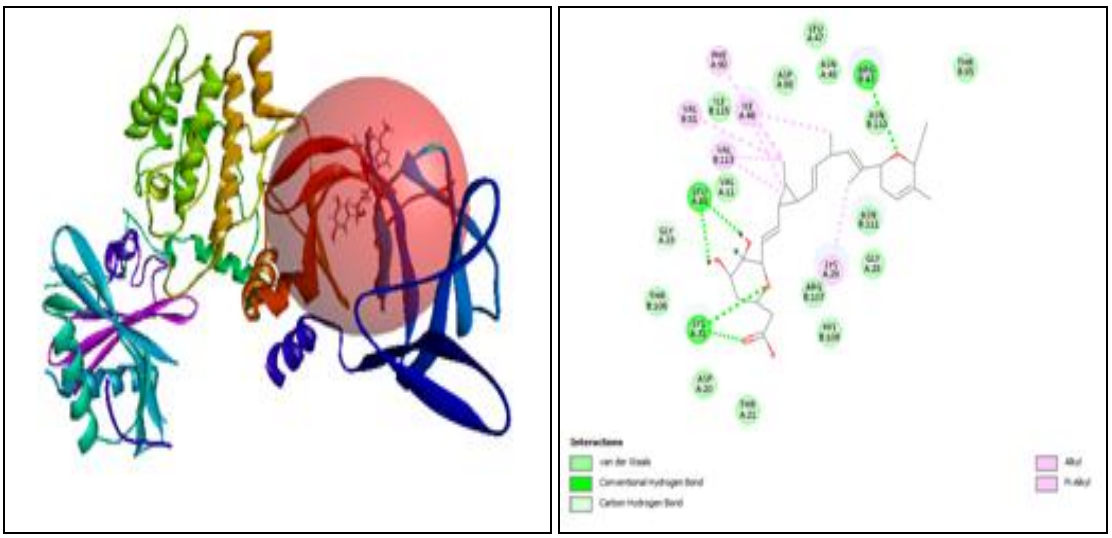

FIG. 2: THE 3D AND 2D PICTURE OF REPROTEROL (25654), COMPLEXED WITH AMPK (PDB ID: 4CFF)

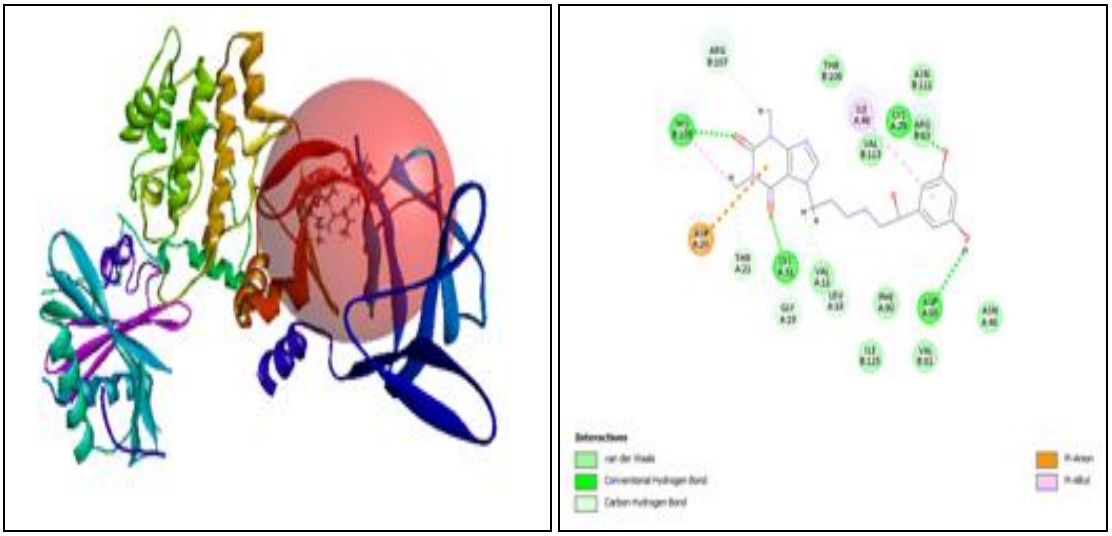

FIG. 3: THE 3D AND 2D PICTURE OF AMBRUTICIN (6918547), COMPLEXED WITH AMPK (PDB ID: 4CFF)

DISCUSSION: The indirect AMPK activators mainly act by modulating those pathways which ultimately enhance the AMP/ATP ratio. Mostly these pathways are ATP-generating catabolic pathways like high physical exercise and Adiponectin secretion by the adipose tissue. Both of them stimulate glucose uptake, and fatty acid oxidation, in muscle cells and enhance insulin sensitivity. Caloric restriction modulates hormones like leptin, ghrelin, and glucagon, which indirectly activate AMPK by modulating cellular energy metabolism.

The glucagon stimulates glycogenolysis and gluconeogenesis in the liver, leading to an increase in the AMP: ATP ratio. The metabolic stresses, like hypoxia, ischemia, and nutrient deprivation, can activate AMPK as a protective mechanism to maintain cellular energy homeostasis and promote cell survival under adverse conditions. Some of the AMP-mimetic compounds mimic the effects of AMP by either inhibiting ATP production or promoting ATP consumption, leading to an increase in the AMP:ATP ratio.

They include inhibitors of mitochondrial ATP synthesis, such as oligomycin. Another possibility could be the allosteric activation of existing AMPK protein in that cell, known as direct activation. Further, it is important to note that the activation of AMPK is tissue-specific. For example, leptin activates AMPK in adipose tissue, smooth muscles (SM), and the liver but inhibits it in the hypothalamus [12]. Similarly, adiponectin and anti-diabetic drugs, activate the AMPK in the peripheral tissues. When we talk of food supplements, several secondary metabolites have shown the properties of AMPK activation in different tissues [13]. Therefore selection of AMPK activators as medicine should be considered by specific choice. In the liver, smooth muscle and adipose tissue its activation becomes more important as they are the place of metabolism of lipids and glucose, the main source of energy production. The shilajit is an established herbal drug, which has been in clinical use for centuries, for several therapeutic claims. One of them is the management of metabolic syndrome, which is directly linked to the AMPK/mTOR energy switch.

Earlier publications have tried to search for several indirect and direct AMPK activators, which can be used for the management of overweight/obesity, which is the basic cause behind the pathogenesis of all the diseases of metabolic syndrome. The crystal structure of human AMPK, developed in a complex with small molecule activators, has opened a path for the search of other molecules. They bind at a site between the kinase domain and the carbohydrate-binding module, to stabilize the interaction between these two components [9].

In this process, the AMPK is activated by phosphorylation of a threonine residue (Thr-172) within the activation loop of the kinase domain, which is attributed to the two upstream kinases, i.e. liver kinase B1 (LKB1) and calcium/calmodulin-dependent protein kinase kinase (CaMKKβ). This increased AMPK activation, further increases the phosphorylation of its downstream targets such as

acetyl-CoA carboxylase, involved in energy production, overall leading to a reduction in anabolic pathways (ATP-utilizing) and an increase in the rate of catabolic pathways (ATP-producing). Under the list of small molecules, which have been identified as direct activators of AMPK, the A-769662 has been extensively studied [27], The Thienopyridone drugs are selective activators of AMP-activated protein kinase beta1-containing complexes [28]. It requires the presence of the carbohydrate-binding module (CBM, also known as the glycogen-binding domain) at the N-terminus of the β-subunit since it shares sequence similarity with a domain found in several proteins that bind carbohydrates. The kinase domain of the α-subunit and the CBM of the β-subunit are connected to their C-terminal scaffold domains by flexible linkers. The AMP is the main cellular molecule for the allosteric activation of AMPK. It triggers the conformational change in the AMPK complex that allows further activation, by phosphorylation of Thr-172 in the AMPKα subunit, allowing it to further interact with the cystathionine-βsynthase domain repeats of the AMPKγ subunit. Two upstream kinases, LKB118 and CaMKKβ (Ca2+/calmodulin-dependent protein kinase β), 19 have been extensively documented to phosphorylate Thr-172 of the AMPKα subunit [12].

Those agents, which can activate AMPK, by modulating the cellular AMP or calcium accumulation are called indirect AMPK activators because they do not directly bind to the AMPK protein. Metformin, a type of biguanide, initially isolated from the plant *Galega officinalis*, is one of them. It is an antidiabetic drug that can reduce hepatic glucose production and enhance peripheral insulin sensitivity. The metformin inhibits complex I of the mitochondrial respiratory chain, resulting in an increased AMP:ATP ratio. Similarly, Thiazolidinediones (TZDs), also known as glitazones, is another example. It primarily activates the peroxisome proliferator activated receptors (PPARγ), resulting in AMP accumulation, again by inhibiting the complex I of the mitochondrial respiratory chain [29].

There are several phytochemicals, found in spices, vegetables, fruits and medicinal plants, which have shown similar indirect AMPK activation. These include resveratrol from red grapes, quercetin, genistein epigallocatechin gallate, berberine, curcumin and Ginsenoside (tetracyclic triterpene glycosides) isolated from Panax ginseng.

They have different mechanisms of action to inhibit mitochondrial ATP production resulting in AMP increase. However, α-Lipoic acid (ALA), another class of molecule, derived from octanoic acid, acts as a cofactor for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase in the TCA cycle, activates AMPK through the increase in the intracellular calcium level in C2C12 myotubes, suggesting that CaMKK, but not LKB1, is responsible for AMPK activation. PT-1 Another small molecule activator of AMPK, PT-1, was initially isolated *via* a screen of compounds that activated the truncated AMPKα1 construct containing only the KD and the AID. PT-1 activates the complete AMPK α1β1γ1, as well as the AMPKα1 KD-AID, construct but not the AMPKα1 KD construct, suggesting that PT-1 directly binds to the cleft between the KD and the AID, thereby relieving autoinhibition. The authors have proposed that the failure of PT-1 to activate γ3-containing complexes in muscle is not an intrinsic feature of such complexes but occurs because PT-1 does not increase cellular AMP:ATP ratios in the distinct subcellular compartments containing γ3-complexes.

Therefore, the molecular details of PT-1 action should be further studied to address the questions raised by these contradictory results. MT 63–78 (Debio0930) another AMPK direct modulator has recently been identified to allosterically activate AMPK. It is highly selective for the AMPK complex containing the AMPKβ1 subunit, as was seen for A-769662 and salicylate. Notably, MT 63–78 strongly suppresses the growth of prostate cancer cell lines with concomitant activation of AMPK but without any significant change in cellular ATP, ADP and AMP levels. A recent screening of a chemical library containing 1,200 AMP mimetics has identified 5-(5-hydroxyl-isoxazol-3-yl)-furan-2- phosphonic acid, termed Compound-2 (C-2), and its pro-drug C-13, as potent allosteric activators of AMPK [27-29].

CONCLUSION: Shilajit, a traditional Ayurvedic herbo-mineral preparation, has been shown to have a significant AMPK activation potential, attributed

to the presence of Reproterol (CID-25654) and Ambruticin (CID-6918547), showing better binding energy, in comparison to standard drug "A-769662 (CID: 54708532)".

This study opens the path for its repurposing as an anti-obesity drug as an "evidence-based medicine", subject to its clinical validation.

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Limitations: Although we have studied only 2 metabolites of shilajit in detail, but similar studies with more metabolites, may give novel leads for more computer-aided drug designing.

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