(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 June 2020 (11.06.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/115765 A1

(51) International Patent Classification:
A61K 9/20 (2006.01) *A61K 31/00* (2006.01)
A61K 9/48 (2006.01)

(21) International Application Number:
PCT/IN2019/050885

(22) International Filing Date:
05 December 2019 (05.12.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
201821045884 05 December 2018 (05.12.2018) IN

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:
— *with international search report (Art. 21(3))*
— *with amended claims and statement (Art. 19(1))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

WO 2020/115765 A1

(54) Title: NOVEL SYNERGISTIC COMPOSITION COMPRISING SIRT1 AND AMPK ACTIVATORS FOR TREATING METABOLIC SYNDROME

(57) Abstract: The present invention herein discloses novel synergistic composition comprising SIRT1 and AMPK activators for treating metabolic syndrome. Particularly the composition comprising therapeutic blend of SIRT1 activator(s) and stabilized AMPK activator(s) present in the ratio of 1: 1 to 1: 5, along with pharmaceutically acceptable excipients. More particularly the invention discloses novel synergistic nutritional composition comprising synergistic combination of N1-methylnicotinamide salt to oxaloacetic acid to standardized ascorbic acid present in the ratio ranges from 1:1:1 to 1:2:2, more particularly 1: 1.1: 1.6. The present synergistic nutritional compositions are useful for treating a subject suffering from metabolic syndrome such as hypertension, obesity, hyperlipidemia and diabetes and other related cardiovascular diseases. Further it improves ATP level and antioxidant activity.

NOVEL SYNERGISTIC COMPOSITION COMPRISING SIRT1 AND AMPK ACTIVATORS FOR TREATING METABOLIC SYNDROME

Technical Field of the invention:

The present invention relates to novel synergistic compositions comprising of sirtuins (SIRT1) activator(s) and 5' adenosine monophosphate-activated protein kinase (AMPK) activator(s) for treating metabolic syndrome. Particularly, the invention relates to synergistic compositions comprising a therapeutically effective amount of SIRT1 activator and 5' AMP-activated protein kinase activator with a bioenhancer along with pharmaceutically acceptable excipients. More particularly, the invention relates to synergistic compositions, wherein SIRT1 activator is N-1-methyl nicotinamide salt, AMPK activator is oxaloaceate and the bioenhancer is an antioxidant particularly ascorbic acid.

The present synergistic nutritional compositions are useful for treating a subject suffering from metabolic syndrome such as hypertension, obesity, hyperlipidemia and diabetes and other related cardiovascular diseases.

Background and Prior art:

Metabolic Syndrome or (MetSyn) is an asymptomatic, pathophysiological state characterized by obesity, insulin resistance, hypertension, dysglycaemia and dyslipidaemia. While several criteria and definitions have been used to classify MetSyn, it is generally agreed that in case of MetSyn a combination of two or more of the following components must be present: large waist circumference, elevated triglycerides, low HDL-cholesterol, raised blood pressure and elevated fasting blood glucose.

According to the National Heart, Lung and Blood Institute (NHLBI), the cluster of metabolic factors involved include: abdominal obesity, high blood pressure, impaired fasting blood glucose, low HDL (good) cholesterol and high triglyceride

levels. The NHLBI and American Heart Association (AHA) recommend a diagnosis of metabolic syndrome when a person has 3 or more of these factors.

There are several factors interconnected which may cause metabolic syndrome. Obesity plus a sedentary lifestyle contributes to risk factors for metabolic syndrome. These include high cholesterol, insulin resistance and high blood pressure. These risk factors may lead to cardiovascular disease and type 2 diabetes. Some experts believe that hormonal changes caused by chronic stress lead to abdominal obesity, insulin resistance, and higher blood lipids (triglycerides and cholesterol). Other factors that may contribute to metabolic syndrome include genetic changes in a person's ability to break down fats (lipids) in the blood; old age; poor nutrition, and problems in how body fat is distributed.

The International Diabetes Federation (IDF) estimates that approximately 25% of the world's population has MetSyn, although this estimate varies widely on the basis of age, ethnicity, and gender of the population studied. Therefore, it is important to find out a better remedy to root out the prevalence of MetSyn components in the population preferably young adults (18-30 years).

Further, metabolic syndrome is characterized by a group of metabolic risk factors, including abdominal obesity, atherogenic dyslipidemia (e.g. high triglyceride levels, low HDL cholesterol levels, and high LDL cholesterol levels), hypertension, insulin resistance, prothrombotic state (e.g. high fibrinogen or plasminogen activator inhibitor-1 levels), and proinflammatory state (e.g. elevated C-reactive protein levels).

It is observed that insulin resistance and metabolic syndrome affect the SIRT1 gene and protein expression in peripheral blood mononuclear cells (PBMCs). Glucose and saturated fatty acids may be implicated in SIRT1 downregulation through induction of oxidative stress and depletion of NAD^+.

Notably, NAD^+ being an essential substrate of sirtuin deacetylation has created renewed interest in NAD^+ as a potential modulator of longevity and better health in human. The sirtuin deacetylation reaction involves the removal of an acetyl group from target substrates via the conversion of NAD^+ to nicotinamide and O-acetyl-ADP-ribose.

Further, phylogenetic analysis of sirtuins across multiple species has led to grouping of enzymes into four main classes (I–IV) of eukaryotic enzymes. According to this classification, SIRT1–3 are part of class I, SIRT4 represents class II, SIRT5 belongs to class III, while SIRT6 and SIRT7 are members of class IV. Sirtuins may also be distinguished by subcellular localization: SIRT1, 6 and 7 are nuclear; SIRT3–5 are mitochondrial; while SIRT2 is mainly cytosolic, but can shuttle to the nucleus during mitosis. However, isoforms of SIRT1 and SIRT5 have also been identified in the cytoplasm. (*Progress in Molecular Biology and Translational Science Volume 154, 2018, Pages 25-69).*

Sirtuins such as SIRT1 are conserved protein NAD^+-dependent deacylases and thus their function is intrinsically linked to cellular metabolism. SIRT1 are supported by their diverse cellular location allowing cells to sense changes in energy levels in the nucleus, cytoplasm, and mitochondrion. SIRT1 plays a critical role in metabolic health by deacetylating many target proteins in numerous tissues, including liver, muscle, adipose tissue, heart, and endothelium.

Sirtuins catalyze NAD^+-dependent protein deacetylation and are critical regulators of transcription, apoptosis, metabolism, and aging. SIRT1 has been implicated as a key mediator of the pathways downstream of calorie restriction that have been shown to delay the onset and reduce the incidence of age-related diseases such as type 2 diabetes.

SIRT1 mediated deaceylation has significant impact on the activity of many proteins, resulting in the regulation of a number of proteins and their translation which play important roles in the biological process including oxidative stress, metabolism, cell proliferation, and genomic stability.

Recently, the role of SIRT1 under normal physiological conditions as well as during disease states has drawn great interest in the research community. There are certain prior arts that deal with different SIRT1 activators for treating aging, metabolic diseases, neurodegenerative diseases, cancer, and cardiovascular dysfunction.

EP2953485B1 discloses *Mangifera Indica* fruit powder for use as a Sirtuin 1 activating agent in preventing, or treating obesity, diabetes type II, elevated blood lipid levels, atherosclerosis and/or cardiovascular diseases.

US20070212395A1 discloses sirtuin-activating agent preferably resveratrol, for treating ocular conditions such as age-related macular degeneration.*WO2018015861A1* discloses use of 1-MNA or a pharmaceutically acceptable salt thereof for reducing the risk of cardiovascular disease such as rheumatoid arthritis, colon cancer, breast cancer, lung cancer, infection, inflammatory bowel disease, lupus erythematosus, pneumococcal pneumonia, rheumatic fever, tuberculosis, renal failure, amyotrophic lateral sclerosis, or a combination thereof.

US8481570B2 relates to 1-methylnicotinamide derivatives and formulations for treatment of lipoprotein abnormalities such as atherosclerosis, hyperlipidaemias, angina pectoris or cardiac risk.

Further *US2009118225A1* discloses 1-methyl nicotinamide and derivatives for treatment of gastric injury.

WO2016066588A1 discloses synergistic compositions for stimulating the expression of Sirtuin comprising combinations of a first component selected from berberine and

tyrosol with a second component selected from quercetin, tyrosol, catechin and ferulic acid.

However, it is found that to maintain the specific metabolic pathways and energy homeostasis, the human body needs additional metabolic activator or regulator that can co-ordinate metabolic pathways and balance the nutrient supply with energy demand.

AMPK functions as a sensor of cellular energy status and as a master regulator of metabolism and energy homeostasis. When ATP levels decrease, AMPK is activated to boost ATP production and to inhibit ATP usage, thus restoring energy balance. Similarly, SIRT1 is activated in response to changes in the energy status to promote transcription of genes that mediate the metabolic response to stress, starvation, or calorie restriction.

AMPK activation is stimulation of hepatic fatty acid oxidation, ketogenesis, stimulation of skeletal muscle fatty acid oxidation and glucose uptake, inhibition of cholesterol synthesis, lipogenesis and triglyceride synthesis, inhibition of adipocyte lipogenesis, activation of adipocyte lipolysis, and modulation of insulin secretion by pancreatic beta-cells.

AMP-activated protein kinase (AMPK) acts as an intracellular metabolic sensor in a variety of cells, where it monitors and responds to variations in the AMP:ATP ratio. Further AMPK is recognized as a major regulator of lipid biosynthetic pathways due to its role in the phosphorylation and inactivation of key enzymes such as acetyl-CoA carboxylase, fatty acid synthase. Further AMPK increases the clearance of blood glucose by facilitating the transport of glucose to the skeletal muscle.

APMK activities are disclosed in some of the prior arts.

US20070244202A1 relates to an AMPK activator containing resveratrol as an active ingredient for regulating lipid metabolism in human.

US8431552B2 reports synergistic combination of AMPK activator, metformin along oxidative phosphorylation inhibitor, an ionophore, anti-inflammatory agent, serotonin activity complex for treating metabolic syndrome.

There are several methods known to activate AMPK, but these methods have some drawbacks associated with them. AMPK can be activated with metformin, resveratrol and acadesine. But metformin may also produce lactic acidosis, which can become a life-threatening condition, especially where a patient has renal insufficiency. Resveratrol has a very low bioavailability, and large amounts may be necessary in order to achieve efficacy. Acadesine or AICAR is banned by the World Anti-Doping Code for athletic events.

Further Resveratrol has been claimed to be an activator of Sirtuin 1, but this effect has been disputed based on the fact that the initially used activity assay, using a non-physiological substrate peptide, can produce artificial results. Resveratrol exhibits multiple off-target activities against receptors, enzymes, transporters, and ion channels. *[The Journal of Biological Chemistry; March 2010;285; 8340-8351].*

In view of above prior arts, it is evident that many active pharmaceutical ingredients function individually because of which the resultant effect for controlling body metabolism becomes less effective and is also accompanied by several side effects. Further the use of natural products as SIRT activators involves optimum extraction to obtain desired phytoconstituents that makes the process tedious.

Therefore, a need arises to develop a dietary composition, where therapeutically active agents function synergistically to maintain energy homeostasis and metabolism so that it can improve organ function, disease resistance, and increased longevity in human.

Several observations support a model where, in response to stress and reduced nutrients, a metabolic pathway is activated within which AMPK and SIRT1 concordantly function to ensure an appropriate cellular response and adaptation to environmental modifications.

The AMPK-SIRT1 or SIRT1-AMPK pathway is an important regulator of energy metabolism and therefore a potential target for prevention and therapy of metabolic diseases. AMP-activated protein kinase (AMPK) and the sirtuins SIRT1 are well-known key sensors of energy status and regulators of glucose and lipid metabolism. They work in a finely tuned network to regulate mitochondrial proliferation and metabolism and energy expenditure. Accordingly, this network appears to be a strong target for prevention and control of metabolic diseases such as atherosclerosis, obesity and diabetes.

AMPK enhances SIRT1 activity by increasing cellular NAD+ levels, resulting in the deacetylation and modulation of the activity of downstream SIRT1 targets.

Therefore, the inventors have successfully developed a synergistic composition based on the mechanism of AMPK-SIRT1 pathway wherein AMPK activator and SIRT1 activator functions concurrently to enhance the NAD level and thus regulating metabolic processes.

Objective of the invention:

The primary object of the invention is to provide a dietary composition for treating metabolic diseases.

Another object of the invention is to provide composition of active ingredients to activate SIRT1 and AMPK enzyme that work synergistically for enhancing NAD level, subsequently ATP enhancement.

Further object of the invention is to provide a synergistic composition of active ingredients to improve glucose, insulin and lipid metabolism, cell protection, energy homeostasis, and thereby maintain the healthy life.

Summary of the invention:

To meet the above objectives, the inventors of the instant invention carried out rigorous experiments to establish the significant effect of enzyme activators that improve the metabolic disorders or syndrome.

In an aspect, the invention relates to synergistic composition of active ingredients for treating metabolic syndrome.

In another aspect, the invention relates to synergistic composition of SIRT1 and AMPK activators for regulating metabolic disorders such as diabetic, obesity, high blood pressure, high blood sugar, triglycerides, low serum high-density lipoprotein (HDL) and other cardiovascular diseases.

In yet another aspect, the invention provides potent synergistic composition comprising combination of SIRT1 and AMPK activators, optionally bioenhancers in an effective amount, wherein SIRT1 activator is N-1-methyl nicotinamide and AMPK activator is oxaloacetate and optionally bioenhancers are antioxidants selected from the group consisting of vitamins, ascorbic acid, sorbic acid, citric acid, propyl gallate and like thereof.

In one more aspect, the invention provides synergistic nutritional composition of protein dietary supplements, wherein the composition is essentially composed of N^1-methyl nicotinamide chloride and stabilized OAA, wherein the ratio of N-1-MNA to stabilized OAA is maintained in the range of 1:1 to 1:5.

In another aspect, the instant invention provides synergistic compositions comprising combination of N-1-methyl nicotinamide and oxaloaceatate in a subject suffering

from metabolic syndrome such as diabetic, high blood pressure, cholesterol disorders, obesity, cardiovascular diseases and like thereof.

In yet another aspect, the invention relates to synergistic compositions comprising combination of N-1-methyl nicotinamide which is present in the range of 1 to 500 mg and oxaloacetate present in the range of 1 to 250 mg, optionally bioenhancers such as antioxidants are present in the range of 1 to 500 mg along with pharmaceutically acceptable excipients/carriers.

In yet another aspect, the invention relates to synergistic composition of N-1-methyl nicotinamide and oxaloacetate, wherein the N-1-methyl nicotinamide allows deacetylation and oxaloacetate allows phosphorylation of the target molecules concurrently to elevate NAD level and subsequently enhance ATP level.

Abbreviations:

OAA: Oxaloacetate

1-MNA: N-1-Methyl Nicotinamide

NAD/NAD+: Nicotinamide adenine dinucleotide

SIRT1: Silent information regulator T1 (sirtuin family)

AMPK: 5' adenosine monophosphate-activated protein kinase

NAM: Nicotinamide

NNMT: Nicotinamide N-methyltransferase

ATP: Adenosine triphosphate

Brief description of figures:

Fig. 1 depicts cytotoxic effect of test substances on Human Hepatocyte cells

Fig. 2 depicts effect of test substances on cellular ATP concentration in treated groups

Fig. 3 depicts effect of test substances in the improvement of cellular ATP levels over control

Detailed Description of the invention:

The invention will now be described in detail in connection with certain preferred and optional embodiments, so that various aspects thereof may be more fully interpreted and comprehended. However, any skilled person or artisan will appreciate the extent to which such embodiments could be generalized in practice.

It is further to be understood that all terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting in any manner or scope.

Unless defined otherwise, all technical and scientific expressions used herein have the same meaning as commonly understood by one of ordinary skill in the art to which embodiments of the invention pertain.

In preferred embodiment, the present invention provides synergistic composition of therapeutically active agents.

Particularly, the invention provides synergistic combination of SIRT1 activator and AMPK activator, optionally antioxidant as bioenhancer in therapeutically effective amount.

In another preferred embodiment, the invention discloses the synergistic combination of SIRT1 and AMPK activators, optionally antioxidant or pharmaceutically acceptable salts thereof along with pharmaceutically acceptable carriers. In one embodiment, the invention provides novel synergistic nutritional composition for treating metabolic syndrome comprising a therapeutic blend of SIRT1 activator(s) and stabilized AMPK activator(s) present in the ratio of 1: 1 to 1: 5.

In describing and claiming the embodiments of the present invention, the following terminology will be used in accordance with the definitions set out below or the definition known in the state of art.

The singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

The term "pharmaceutically-acceptable salts" refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds, as well as solvates, co-crystals, polymorphs and the like of the salts.

In one embodiment, the present invention provides SIRT1 activator for enhancing NAD level, wherein the SIRT activator is 'N-1-methyl nicotinamide' (1-MNA). 1-MNA can also be referred as N(1)-Methylnicotinamide; N^1-Methylnicotinamide; 1-methylnicotinamide cation;3-(aminocarbonyl)-1-methylpyridinium; trigonellinamide; 1-methyl-3-carbamoylpyridinium; 1-methyl-3-carbamoylpyridinium cation; 3-amido-n-methylpyridinium: 1-methyl-3-pyridinecarboxamide; 3-carbamoyl-1-methyl-pyridinium; n-methyl-3-carbamidopyridinium; n-methyl-3-carbamoylpyridinium ion; n1-methylnicotinamide; 1-methyl-3-carbamoylpyridinium; 1-methyl-3-carbamoylpyridinium cation; 3-amido-n-methylpyridinium; 1-methyl-3-pyridinecarboxamide; 3-carbamoyl-1-methyl pyridinium; n-1-methylnicotinamide; n-methyl-3-carbamidopyridinium; n-methyl-3-carbamoylpyridinium ion; n'-methylnicotinamide; n'methylnicotinamide; n1-methylnicotinamide; 1-methylnicotinamide cation; 3-(aminocarbonyl)-1-methylpyridinium; n(1)-methylnicotinamide; and salts thereof.

The salts of N^1-MNA are selected from the group consisting of n(1)-methylnicotinamide chloride; n(1)-methylnicotinamide cyanide; n(1)-methylnicotinamide fluoride; n(1) methylnicotinamide iodide, n(1)-methylnicotinamide methylsulfate; n(1)-methylnicotinamide perchlorate; n(1)-methylnicotinamide bromide; n(1)-methylnicotinamide iodide; n(1)-

methylnicotinamide tetrafluoroborate (1-); trigonellamide chloride; particularly the MNA salt is n(1)-methylnicotinamide chloride.

In metabolic pathways, SIRT1 controls adipocyte cytokines expression, fat cells maturation, insulin secretion, plasma glucose levels, cholesterol and lipid homeostasis, and mitochondrial energy capacity. SIRT1 regulates the lifespan through several mechanisms.

The terms "sirtuin activator" or "sirtuin activating compound" refer to a compound that increases the level of a sirtuin protein and/or increases at least one activity of a sirtuin protein.

Nicotinamide N-methyltransferase (NNMT) methylates nicotinamide is a form of vitamin B3, to produce N-1-methyl nicotinamide (1-MNA). Supplementation of high-fat diet with 1-MNA decreases serum and liver cholesterol and liver triglycerides levels in a subject. 1-MNA levels stabilize sirtuin 1 protein, an effect that is required for their metabolic benefits.

Further, methylation of nicotinamide by NNMT is a major pathway for the clearance of excess vitamin B3 from the body. 1-MNA works in NAD+ biosynthetic pathways, which stimulate Sirt1 activity by increasing NAD+ levels, vitamin B3 clearance pathways also regulate hepatic nutrient metabolism through MNA-mediated Sirt1 protein stabilization.

The present inventors found beneficial effects of dietary 1-MNA supplementation in a subject; where 1-MNA increased SIRT1 protein expression *in vivo*. In line with the known metabolic effects of Sirt1, 1-MNA significantly lowered liver cholesterol and triglyceride levels, while also suppressing fatty acid and cholesterol synthesis and the expression of lipogenic and cholesterol synthesis genes and bile acid transport. Further 1-MNA or its derivatives are useful for lowering the LDL levels in humans.

In another embodiment, the synergistic composition comprises therapeutically effective amount of 1-MNA or pharmaceutically acceptable salts thereof, wherein 1-MNA is present in the range of 1-500 mg, preferably in the range of 1-250 mg of total composition.

In another embodiment, the invention provides AMPK activator for synergistically enhancing NAD level, wherein the AMPK activator is 'Oxaloaceate' (OAA).

'OAA' can also be referred as oxobutanedioate; 2-oxobutanedioate; keto-oxaloacetate; oxosuccinate; oxaloacetate dianion and pharmaceutically acceptable salts or isomers thereof.

According to another embodiment of the present invention there is provided a method of preventing and treating metabolic syndrome in a mammal, comprising administration of a therapeutically effective amount of OAA to activate AMPK.

The inventors found that AMPK activation increases SIRT1 expression in macrophages and that this led to the deacetylation and down regulation of biomarkers related to metabolic diseases.

At higher pH, oxaloacetic acid in water occurs in three forms, 1) the hydrated form, 2) a keto form, and 3) an enol form. Enol-oxaloacetate is converted to keto-oxaloacetate with the enzyme enol-keto tautomerase, a ubiquitous enzyme throughout the human body. In the instant composition OAA compound is stabilized in presence of bioenhancers. In another embodiment, the bioenhancers are antioxidant and selected from group consisting of vitamins, ascorbic acid, sorbic acid, citric acid, propyl gallate and like thereof, wherein the antioxidant is present in the range of 1-500 mg, preferably in the range of 1-300 mg of total composition.

According to the invention, the stabilized oxaloacetate is more stable and bioavailable form of OAA, the antioxidant not only stabilizes the oxaloacetate but also improves it in vivo absorption.

In one embodiment, the active moiety OAA is stabilized by specific amount of ascorbic acid, wherein the ratio of OAA to ascorbic acid is ranged from 1:1 to 1:2, preferably 1:1.45 to 1:1.55. Particularly ascorbic acid or vitamin C is obtained from natural source extract. The extract is further standardized to get higher concentration of ascorbic acid.

It is observed that oxaloacetic acid when administered orally, gets moved from the blood stream and taken into individual cells within few minutes. Within the cells the oxaloacetate reacts into L-malate as it encounters the enzyme malate dehydrogenase (MDH). MDH is ubiquitous in human cells, and the conversion of oxaloacetate to malate is very energetically favorable. As oxaloacetate is converted to malate, NADH is converted to NAD+. The resulting increase in the NAD+/NADH ratio leads to the phosphorylation (activation) of AMPK. There is evidence that the NAD+/NADH ratio specifically activates SIRT1 production, which may be a primary signal for activating SIRT1.

The activation of AMPK results in therapeutic effect for improving metabolic functions. In the liver, there is decreased glucose output and improvement in glucose homeostasis, decreased fatty acid and cholesterol synthesis and increased fatty acid oxidation. A reduction in the ability to store fat, due to the down-regulation of fatty acid synthesis, results in long-term weight reductions. The combinations of all these effects are an excellent treatment for metabolic syndrome, diabetes and obesity.

AMPK is a fuel-sensing enzyme that is activated by decreases in a cell's energy state as indicated by an increased AMP/ATP ratio. When activated, it initiates metabolic and genetic events that restore ATP levels by stimulating processes that generate ATP (e.g., fatty acid oxidation) and inhibiting others that consume ATP but are not acutely required for survival (e.g., triglyceride and protein synthesis, cell proliferation).

In another embodiment, the invention provides synergistic effect of SIRT1 and AMPK activators for controlling metabolic syndrome.

It is noteworthy that SIRT1 and AMPK activators, synergistically work to elevate the NAD level in the blood and thus ameliorating metabolic syndrome through ATP. The joint activation of SIRT1 and AMPK allows for the concurrent deacetylation and phosphorylation of the target molecules and results in decreased susceptibility to metabolic syndrome-associated disorders.

Further the inventors demonstrate that AMPK controls the expression of genes involved in energy metabolism in a subject by acting in coordination with another metabolic sensor, the NAD+-dependent type III deacetylase SIRT1. AMPK enhances SIRT1 activity by increasing cellular NAD+ levels, resulting in the deacetylation and modulation of the activity of downstream SIRT1 targets. The AMPK-induced SIRT1-mediated deacetylation of these targets exhibit therapeutic effects of AMPK and SIRT1 on energy metabolism.

Interestingly, the inventors further demonstrate that down regulation of SIRT1 or AMPK in human body is a predisposition to the metabolic syndrome and accelerated aging.

In another embodiment, the synergistic composition comprises therapeutically effective amount of OAA or pharmaceutically acceptable salts thereof, wherein OAA is present in the range of 1-250 mg, preferably in the range of 1-200 mg of total composition.

In additional embodiment, the invention provides synergistic composition comprising antioxidant as bioenhancer, wherein the antioxidant is selected from the group consisting of butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid, ascorbic acid, and sodium bisulfate, vitamin E, A, D and like thereof.

In another embodiment, the invention discloses use of antioxidant in the instant composition, wherein antioxidant supplement is accompanied by an increase in serum

NAD^+ levels, in both oxidised and reduced NAD^+ and thus increasing the efficiency of electron transfer and ATP production in the cell.

The addition of antioxidant in effective amount enhances the therapeutic efficacy of instant composition for treating metabolic syndrome.

In another embodiment, the invention relates to synergistic composition which is useful for treating metabolic syndrome.

The 'metabolic syndrome' represents a collection of factors, such as hypertension, obesity, hyperlipidemia and diabetes among others, associated with increased risk for cardiovascular disease. Metabolic syndrome is becoming increasingly common, largely as a result of the increase in the prevalence of obesity. The term metabolic syndrome is interchangeable with 'metabolic disorders' or metabolic diseases or dysfunction in metabolic pathway as mention in the present invention. Metabolic syndrome (also known as "syndrome X," "dysmetabolic syndrome," "obesity syndrome," "Reaven syndrome" and interchangeably referred to herein as the "syndrome") has emerged as a growing problem. Metabolic syndrome refers to cluster of conditions such as increased blood pressure, high blood sugar, excess body fat around the waist, obesity, and abnormal cholesterol or triglyceride levels that occur together, low serum high-density lipoprotein (HDL) that increase the risk of heart disease, stroke and diabetes.

In the context of the present invention, the terms "treatment or treating" and the like refers to alleviating, slowing the progression, prophylaxis, preventing, attenuating, curing or ameliorating at least one symptom /syndrome of the condition or disease related to disruption of metabolic pathways or their regulation.

The instant composition is used for treating metabolic syndrome in a subject in need thereof, means either the administration of the remedy to prevent the onset or

occurrence of metabolic disorders, or the treatment of already existed metabolic disorders.

The term 'subject in need thereof' pertains to subject preferably mammal, more preferably human suffering from metabolic disorders or in a subject to prevent occurrence of metabolic diseases.

The phrase "therapeutically-effective amount" means that amount of such a substance that produces some desired local or systemic effect at a reasonable benefit/risk ratio applicable to any treatment. The therapeutically effective amount of such substance will vary depending upon the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art.

For example, certain compositions described herein may be administered in a sufficient amount to produce a desired effect on metabolic disorders or complications thereof, at a reasonable benefit/risk ratio applicable to such treatment.

Thus, a "therapeutically effective" amount is an amount that reduces the risk, potential, possibility or occurrence of a disease or disorder, or provides some alleviation, mitigation, and/or reduction of at least one indicator (e.g., blood or serum CRP level), and/or decrease in at least one clinical symptom of a disease or disorder (e.g., metabolic syndrome such as diabetic or obesity as disclosed herein).

In one more embodiment, the synergistic composition comprising combination of N^1-methyl nicotinamide chloride and oxaloaceatate present in the ratio of 1:1 to 1:5, particularly in the ratio of 1:1 to 1:1.5; wherein the ratio of N^1-methyl nicotinamide chloride to stabilize oxaloacetate is 1:1 to 1:5, particularly in the ratio of 1: 1 to 1: 3.0 with respect to total composition.

In further embodiment, the stabilized oxaloacetate comprises homogenous premix of crystalline organic oxaloacetic acid with standardized ascorbic acid or vitamin C as antioxidant, wherein oxaloacetic acid to antioxidant ratio is in the range of 1:1 to 1:2, particularly in the range of 1: 1.45 to 1:1.75. Further the standardized ascorbic acid comprises 10-250 mg of ascorbic acid.

The term standardized ascorbic acid does not limit the scope of the invention to particular plant extract but also involves certain natural ingredients such as flavanoids, tannins, alcohol, carotenoids, citrus food extract and other fruit extracts.

In yet another embodiment, the invention relates to synergistic compositions comprising combination of N^1-methyl nicotinamide chloride, which is present in the range of 1 to 500 mg; and oxaloaceatate present in the range of 1 to 250 mg and antioxidant present in the range of 1 to 500 mg along with pharmaceutically acceptable excipients/ carriers.

Particularly, the instant synergistic compositions comprise combination of N-1-methyl nicotinamide chloride present in the range of 1 to 250 mg and oxaloaceate present in the range of 1 to 200 mg and antioxidant 1 to 300 mg along with pharmaceutically acceptable excipients / carriers.

In one embodiment, the synergistic composition comprises 15-30%w/w of N-1-methyl nicotinamide chloride; 15-35%w/w of oxaloacetate or oxaloacetic acid and 25-50% w/w of standardized ascorbic acid of total composition.

In one embodiment, the present novel synergistic nutritional composition comprises N1-methylnicotinamide salt to oxaloacetic acid to standardized ascorbic acid in the ratio ranging from 1:1:1 to 1:2:2.In one embodiment, the present invention provides novel synergistic nutritional combination of N1-methylnicotinamide chloride and oxaloacetate and standardized ascorbic acid in the ratio of 1: 1.1: 1.6.

In another embodiment, the instant synergistic composition or a pharmaceutically acceptable salt thereof can be formulated in various pharmaceutically compositions for administration. Non-limiting exemplary pharmaceutical compositions include solutions, suspensions, emulsions, tablets, pills, pellets, powders, multi-particulates, capsules, capsules containing liquids, capsules containing powders, capsules containing multi-particulates, lozenges, sustained-release formulations, suppositories, transdermal patches, transmucosal films, sublingual tablets or films, aerosols, sprays, or any other form suitable for use.

In certain embodiments, the instant composition or a pharmaceutically acceptable salt of the present disclosure is delivered in a controlled-release system or sustained-release system or immediate release system.

In general, the above compositions may be prepared in a conventional manner using conventional pharmaceutically acceptable excipients, using standard techniques known to those skilled in the art of pharmacy.

The pharmaceutically acceptable excipients include but are not limited to diluents, disintegrating agents, binding agents, lubricating rents, sweetening agents, flavoring agents, coloring agents and preservatives.

In yet another embodiment, the synergistic composition of the instant invention may be employed in the treatment of any condition associated with metabolic syndrome such as increased blood pressure, high blood sugar, excess body fat around the waist, obesity, and abnormal cholesterol or triglyceride levels, low serum high-density lipoprotein (HDL) that increase the risk of heart disease, stroke and diabetes and like thereof.

In yet another embodiment, the present synergistic nutritional composition, is useful in liver health wherein the composition decreases glucose output and fatty acid and

cholesterol synthesis, improves glucose homeostasis, increases fatty acid oxidation. It further improves cellular energy and ATP turnover.

As used herein, the term "specific or effective amount" is intended to mean the therapeutically effective dose of instant bioactive compounds namely N^1MNA salts and stabilized OAA in combination to give significant therapeutic efficacy, which is otherwise not obtained by use of single ingredient of the composition.

The term "pharmaceutically acceptable salt" refers to a salt prepared from pharmaceutically acceptable non-toxic acids or bases, metal ions, minerals, chelates, complex, esters, oxide, amines which are well known in the art.

As used herein, the term "pharmaceutically acceptable carriers/vehicles / diluents or excipients" is intended to mean, without limitation, any adjuvants, carriers, excipients, sweetening agents, diluents, preservative, dye/colorants, flavor enhancers, surfactants, wetting agents, dispersing agents, suspending agents, complexing agents, stabilizers, isotonic agent, solvent, emulsifier, encapsulating agent, polymers, coating agent, wax, encapsulating polymeric delivery systems. Excipients may also include, anti-adherents, antioxidants, binders, pH-modifier, solvents, coatings, compression aids, disintegrants, emollients, fillers (diluents), film formers, fragrances, glidants (flow enhancers), lubricants, preservatives, sorbents, anticaking agent, food additives, or waters of hydration.

In an embodiment of the invention, the diluents are selected from starches, hydrolyzed starches, and partially pregelatinized starches, anhydrous lactose, cellulose powder, lactose monohydrate, and sugar alcohols such as sorbitol, xylitol and mannitol, silicified microcrystalline cellulose, ammonium alginate, calcium carbonate, calcium lactate, dibasic calcium phosphate (anhydrous/ dibasic dehydrate/ tribasic), calcium silicate, calcium sulfate, cellulose acetate, corn starch, pregelatinized starch, dextrin, β-cyclodextrin, dextrates, dextrose, erythritol, ethylcellulose, fructose, fumaric acid, glyceryl palmitostearate, magnesium carbonate,

magnesium oxide, maltodextrin, maltose, medium-chain triglycerides, polydextrose, polymethacrylates, sodium alginate, sodium chloride, sterilizable maize, sucrose, sugar spheres, talc, trehalose, xylitol, vehicles like petrolatum, dimethyl sulfoxide and mineral oil or the like. The amount of diluent in the pharmaceutical composition/formulation of the present invention ranges from 1 % to 35% by wt. of the composition/formulation.

In further embodiment, the binder is selected from disaccharides such as sucrose, lactose, polysaccharides and their derivatives like starches, cellulose or modified cellulose such as microcrystalline cellulose and cellulose ethers such as hydroxypropyl cellulose (HPC); hydroxypropyl methyl cellulose (HPMC); sugar alcohols such as xylitol, sorbitol or mannitol; protein like gelatin; synthetic polymers such as polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), starch, acacia, agar, alginic acid, calcium carbonate, calcium lactate, carbomers, carboxymethylcellulose sodium, carrageenan, cellulose acetate phthalate, chitosan, copovidone, corn starch, pregelatinized starch, cottonseed oil, dextrates, dextrin, dextrose, ethylcellulose, guar gum, hydrogenated vegetable oil type I, hydroxyethyl cellulose, hydroxymethyl cellulose hydroxyethylmethyl cellulose, hydroxypropyl cellulose, inulin, cellulose, methyl cellulose, polyvinylpyrrolidone and polyethylene glycol, lactose, liquid glucose, hypromellose, magnesium aluminum silicate, maltodextrin, maltose, methyl-cellulose, microcrystalline cellulose, pectin, poloxamer, polydextrose, polymethacrylates, povidone, sodium alginate, stearic acid, sucrose, sunflower oil, various animal vegetable oils, and white soft paraffin, paraffin, flavorants, colourants and wax. The amount of binder in the pharmaceutical composition/formulation of the present invention ranges from 0.1% by wt. to 10% by wt. of the composition/formulation.

Further, in one embodiment of the invention, the lubricant is selected from magnesium stearate, zinc stearate, calcium stearate, glycerin monostearate, glyceryl behenate, glyceryl palmitostearate, hydrogenated castor oil, hydrogenated vegetable

oil type, light mineral oil, magnesium lauryl sulfate, medium-chain triglycerides, mineral oil, myristic acid, palmitic acid, poloxamer, polyethylene glycol, sodium benzoate, sodium chloride, sodium lauryl sulfate, sodium stearyl fumarate, stearic acid, talc, potassium benzoate or the like. The amount of lubricant in the pharmaceutical composition/formulation of the present invention ranges from 0.1% by wt. to 5% by wt. of the composition/formulation.

In some embodiment, the glidant is selected from colloidal silicon dioxide, magnesium stearate, fumed silica (colloidal silicon dioxide), starch, talc, calcium phosphate tribasic, cellulose powdered, hydrophobic colloidal silica, magnesium oxide, magnesium silicate, magnesium trisilicate, silicon dioxide or the like. The amount of glidant in the pharmaceutical composition/formulation of the present invention ranges from 0.1% by wt. to 5 % by wt. of the composition/formulation.

In one embodiment, the solvent is selected from water, alcohol, isopropyl alcohol, propylene glycol, mineral oil, benzyl alcohol, benzyl benzoate, flavoresglycol, carbon dioxide, castor oil, corn oil (maize), cottonseed oil, dimethyl ether, albumin, dimethylacetamide, ethyl acetate, ethyl lactate, medium-chain triglycerides, methyl lactate, olive oil, peanut oil, polyethylene glycol, polyoxyl, castor oil, propylene carbonate, pyrrolidone, safflower oil, sesame oil, soybean oil, sunflower oil, water-miscible solvents, organic polar or non-polar solvents or mixtures thereof. The amount of solvent in the pharmaceutical composition/formulation of the present invention is used in a quantity sufficient to 100% by wt. of the composition/formulation.

The additional additives include polymer, a plasticizer, a sweetener, and a powdered flavor, preservative, colorant, surfactant and other excipients. The powdered flavor composition includes a flavourant associated with a solid carrier. Coating materials like synthetic polymers, shellac, corn protein zein or other polysaccharides, gelatin,

fatty acids, waxes, shellac, plastics, and plant fibers and like thereof are also used. The additives are used in the range of 1 to 50 % w/w of unit dose.

Further optionally the antioxidant is selected from vitamins such as vitamin A, vitamin E, vitamin C, alpha tocopherol, amino acid or its derivatives such as lipoid acid, uric acid, carotenoids and like thereof and which are present in the range of 0.1 to 5% w/w of unit dose.

In the context of the present invention, the terms "treatment" and the like refer to alleviate, mitigate, prophylaxis, attenuate, manage, regulate, modulate, control, minimize, lessen, decrease, down regulate, up regulate, improve, moderate, prevent, inhibit, stabilize, ameliorate or cure, heal the indications of metabolic disorders. The treatment further includes delaying or reversing or preventing or reducing the development or progression or formation or occurrence of conditions or indications related to metabolic disorders and /or metabolic diseases and /or metabolic syndrome or metabolic disruption or metabolic dysfunction.

Notably, the instant synergistic composition is non-hazardous, non-toxic and safe for human consumption without any side effects, therefore the instant composition can also be used under preventive therapy in healthy subjects.

The present nutritional composition is used to maintain proper metabolic function in the subject in need thereof, means of administration of the remedy either to prevent occurrence or pre-existing cause of metabolic disorders such as obesity and hyperlipidemia.

In another embodiment, the invention provides a method of treating a subject suffering from metabolic dysfunctions or disorders or diseases, the method comprising administering to the subject an effective amount of the present synergistic nutritional composition to enhance the metabolic function.

More particularly the present nutritional composition is safe and effective for reducing body weight, triglyceride and cholesterol levels, and thus decreasing the risk of obesity and its fat metabolism related disorders.

The 'subject in need thereof' pertains to subject preferably mammal, more preferably human having pre-existing or onset symptoms of metabolic disorders, like obesity, hyperlipidemia. The subject can be healthy person and use the composition under preventive therapy.

The therapeutically effective amount of the active ingredients or nutrients may be varied depending upon the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art.

Particularly "therapeutically effective" amount is an amount that reduces the risk, potential, possibility or occurrence of a disease or disorder, or provides advanced alleviation, mitigation, and/or reduction or restoration of at least one indicator/biomarker (e.g., blood or serum CRP level), and/or decrease in at least one clinical symptom of metabolic disorders (e.g. obesity, diabetes).

A skilled artisan can determine a pharmaceutically effective amount of the inventive compositions by determining the unit dose. As used herein, a "unit dose" refers to the amount of inventive composition required to produce a response of 50% of maximal effect (i.e. ED50).

In some embodiments, the invention provides method of treating metabolic disorders by administering the present synergistic nutritional composition comprising combination of N^1- MNA and stabilized OAA present in the ratio of 1:1 to 1:5.

The present composition is administered parenterally, orally, topically, buccally, sublingually, transdermally, subcutaneously, intramuscularly, via a medical device, via a stent, by inhalation or via injection.

Therapeutic (prescription) supplements are generally administered by the oral or parenteral or nasal routes for treating metabolic disorders. The therapeutic administration of materials of the present invention may be in conjunction with other therapies.

Further, the instant synergistic nutritional composition can be administered to subject in need thereof, in a form suitable for oral use, such as a tablet, capsule (in the form of delayed release, extended release, sustained release, enteric coated release); hard gelatin capsules, soft gelatin capsules in an oily vehicle, granulate for sublingual use, effervescent tablets, aqueous or oily solution, suspension or emulsion, encapsulate, matrix, coat, beadlets, nanoparticles, caplet, granule, particulate, agglomerate, spansule, chewable tablet, lozenge, troche, solution, suspension, rapidly dissolving film, elixir, gel, as tablets, pellets, granules, capsules, lozenges, aqueous or oily solutions, suspensions, emulsions, sprays or reconstituted dry powdered form with a liquid medium or syrup; for topical use including transmucosal and transdermal use, such as a cream, ointment, gel, aqueous or oil solution or suspension, salve, parch or plaster; for nasal use, such as a snuff nasal spray or nasal drops; for vaginal or rectal use, such as a suppository; for administration by inhalation, such as a finely divided powder or a liquid aerosol; for sub-lingual or buccal use, such as a tablet or capsule, film Further the composition can be formulated for parenteral use including intravenous, subcutaneous, intramuscular, intravascular, infusion, intraperitoneal, intracerebral, intracerebroventricular, or intradermal formulations.

In yet another embodiment, the instant composition is useful for treating metabolic syndrome or energy-related dysfunctions, defective energy metabolism, depletion in cellular energy levels significantly higher increase in glucose uptake, decreased

fatty acid and cholesterol synthesis and increased fatty acid oxidation as compared to individual ingredients.

Additionally, it also controls diseases like renal, hepatic disorder, diabetes, heart disease, endurance performance, neurodegenerative diseases.

In another embodiment, the present invention provides synergistic bioactive composition, which is useful for treating pregnancy complications such as obesity, weight gain, hyperlipidemia, coronary heart diseases, diabetes, hepatic and renal disorders.

In a further embodiment, the invention relates to method for treating metabolic disorders in a subject in need thereof by administering the present synergistic composition in effective oral dosage form wherein the unit dose is formulated in the range of 10-500 mg, which can be administered once or twice or thrice a day based on the indications.

The invention may be further illustrated by the following examples, which are for illustrative purposes only and should not be construed as limiting the scope of the invention in anyway.

This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended claims, and all changes or alterations which come within the ambit of equivalency are intended to be encompassed therein.

Example: 1

i. Composition 1: Synergistic Blend

Ingredient	w/w% unit dose
N^1-Methyl Nicotinamide	15-30
Oxaloacetic acid	15-30
Standardized Ascorbic acid	30-50

Composition 1 refers as therapeutic blend, which can be used in the preparation of present invention formulations.

ii. Composition 1a: Synergistic Blend

Ingredient	w/w% unit dose
N^1-Methyl Nicotinamide	26± 0.5
Oxaloacetic acid	29 ± 0.5
Standardized Ascorbic acid	44 ± 0.5

Therapeutic/ Synergistic blend of N^1-Methyl Nicotinamide- 26± 0.5 % + Oxaloacetic acid- 29 ± 0.5% + Standardized Ascorbic acid- 44 ± 0.5 % is registered under Trademark OXASIRT1™.

iii. Composition 2: Tablet / Capsule

Ingredient	w/w% unit dose
N^1-Methyl Nicotinamide	18± 0.5

Oxaloacetic acid	20± 0.5
Standardized Ascorbic acid	30± 0.5
Excipients	30-35

iv. Composition 3: Tablet / Capsule

Ingredient	**w/w% unit dose**
N^1-Methyl Nicotinamide	15-30%
Oxaloacetic acid	15-35%
Standardized Ascorbic acid	30-50%
Diluent	1-35%
Binder	0.5-5%
Glidant	0.5-5%
Lubricants	0.5-5%
Additives	1-10%
Solvent	QS

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v. Composition 4: Tablet / Capsule

Ingredient	**mg per unit dose**
N^1-Methyl Nicotinamide	45
Oxaloacetic acid	50
Standardized Ascorbic acid	75
Excipients	50-100
Average weight	**220-260 mg**

vi. Composition 5: Tablet / Capsule

Ingredient	**mg per unit dose**
N^1-Methyl Nicotinamide	45
Oxaloacetic acid	50
Standardized Ascorbic acid	75
Microcrystalline Cellulose	5-15
Silicon dioxide	5-15
Hydroxypropyl Methylcellulose	2-10
Zinc Stearate	2-10
PVP K-30	5-10
Talc	1-10
Polysorbate 80	5-20
Manitol	5-20

IPA	QS
Water	QS
Average weight	**220-260 mg**

The present composition is stable for 06 months under the accelerated condition [40°C, 75% RH], where the purity of the active ingredients is above 97%.

Example 2: Hydrophilic Oxygen Radical Absorbance Capacity (ORAC) Test

The Oxygen Radical Absorbance Capacity (ORAC) assay is a method that measures the antioxidant capacity of a substance. The ORAC assay measures a fluorescent signal from a probe that is quenched in the presence of Reactive Oxygen Species (ROS). Addition of an antioxidant absorbs the generated ROS, allowing the fluorescent signal to persist. Trolox® (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) is a vitamin E analogue and a known antioxidant. It is used as a standard by which all unknown antioxidants are compared. Modifications of the ORAC assay include the use of fluorescein as the fluorescent probe (ORACFL), the separation of hydrophilic and lipophilic antioxidants to obtain total antioxidant capacity and an adaptation to a high-throughput platform.

In this analysis, test samples were divided into (03) groups.

Group 1 (G1) served as such stabilized OAA containg homogenous blend of Oxaloacetic acid and Ascorbic acid in the ratio of 1:1.5;

Group 2 (G2) served as such N^1-Methyl Nicotinamide Chloride, and

Group 3 (G3) served as composition 1a.

Experiment:

a) Preparation of test solutions:

10 mg of each test samples were dissolved, and their volume was made up to 10 ml with phosphate buffer. The test samples G1, G2 and G3 were found to be approximately > 95% soluble upon visual inspection. The samples were filtered and the filtrate was used in the assay. For calculation purposes the samples were considered to be 100% soluble. Further dilutions of the filtrates were made as required with phosphate buffer.

b) Trolox (standard) preparation:

For Trolox, a stock solution of 1000µg/ml was prepared. 2mg of Trolox was dissolved in 1000µl of phosphate buffer.

Procedure:

This assay was performed as per Davalos et al *[J. Agric. Food Chem. 52(1): 48-54].* A pre-incubation mixture contained sodium phosphate buffer / Trolox / Test solution of various concentrations and sodium fluorescein. The pre-incubation mixture was placed in a plate, mixed and pre-incubated. Following pre-incubation, 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was added and mixed. The reaction was carried out and the fluorescence measurements were taken at 485nm excitation and 520nm emission filters with the following settings:

1. Mode: Fluorescence intensity
2. Filters: Excitation 485nm, emission 520nm
3. Plate used: Costar 96, Code 3792 from Corning

Results:

ORAC values (Hydrophilic) for G1, G2 and G3 samples were found to be 183±10µM, 119±17µM and 252± 29 µM of Trolox equivalent per gram, respectively. The present composition 1a shows better antioxidant activity.

Table 1: ORAC test results

Table 1: Hydrophilic ORAC values of tested sample

Sample	Conc.	Net AUC (Mean ± SEM)	Average ORAC Value** (Mean ± SEM)
Trolox (Standard)	10µM	440797 ± 26801	Not Applicable
	20µM	685545 ± 94121	
	40µM	1195408 ± 92825	
	80µM	2012089 ± 103441	
	100µM	2395989 ± 99719	
G1	25µg/ml	1522860± 35482	183±10
	12.5µg/ml	1011152 ± 51486	
	6.25µg/ml	712250 ± 48559	
	3.125µg/ml	419984 ± 37610*	
	1.5625µg/ml	242088 ± 18617*	
	0.78125µg/ml	188431 ± 26818*	
G2	25µg/ml	1219848 ± 36402	159±17
	12.5µg/ml	805533 ± 47375	
	6.25µg/ml	456572 ± 47430	
	3.125µg/ml	272454 ± 21887*	
	1.5625µg/ml	135857 ± 23974*	
	0.78125µg/ml	62478 ± 18568*	
G3	25µg/ml	2412487071 ± 32829	252±29
	12.5µg/ml	1530208 ± 52190	
	6.25µg/ml	862233 ± 53082	
	3.125µg/ml	479936 ± 1821*	
	1.5625µg/ml	266296 ± 51030*	
	0.78125µg/ml	114160 ± 38027*	

Example: 3

***In vitro* evaluation of test substances potential on cellular energy by measuring cellular ATP levels in human hepatocyte cell line**

The test substances were evaluated for their *in vitro* effect on cellular energy by measuring cellular ATP levels in human Hepatocytes (HepG2) cell line. In the given experimental conditions, treatment with the test substances improved cellular ATP

levels *in vitro. [J Immunol Methods; 1986; 89: 271-277]; [Proc Natl Acad Sci USA; 2009; 106: 15651–15656].*

Procedure

i. Outline of the method

The *in vitro* cytotoxicity was performed on HepG2 (Human Hepatocyte) cell line to find a nontoxic concentration of the test substances by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and evaluated their potential on cellular respiration by measuring cellular ATP levels.

ii. Preparation of test solution

About 10mg of all the test substances were separately dissolved with 100µl of Dimethyl sulfoxide (DMSO) and the volume was made up with Dulbecco's Modified Eagle Medium – High glucose (DMEM-HG) supplemented with 2% inactivated Fetal Bovine Serum (FBS) to obtain a stock solution of 1 mg/ml concentration and sterilized by 0.22µ syringe filtration. Serial two-fold dilutions were prepared from the stock for carrying out further studies.

iii. Cell line and culture medium:

HepG2 Cell line was cultured in DMEM-HG supplemented with 10% inactivated FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 well microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

iv. Cytotoxicity studies

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 X 105cells/ml using Ham's F12 nutrient mixture containing 10% FBS to obtain a

diluted cell suspension. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once with medium and 100 µl of different concentrations of test substances were added. The plate was then incubated at 37° C for 72 hours in a 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24hour time interval.

a. MTT Assay

After 72 hours of incubation, the drug solutions in the wells were discarded and 50 µl of MTT in PBS (Phosphate Buffer Saline) was added to each well. The plate was gently shaken and incubated for 3 hours at 37°C in a 5% CO_2 atmosphere. The supernatant was removed and 100 µl of 2-propanol was added and the plate was gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the standard formula. The concentration of test substances needed to inhibit the growth of the cell by 50% i.e. CTC_{50} values against cancer cells were generated from the dose-response curves.

v. Estimation of cellular respiration by measuring cellular ATP levels

HepG2 cells were trypsinized from stock culture flasks and the cell count was adjusted to 1.0 X105 cells/ml, the cell suspension was seeded into a 96-well plate. After 24 hours, cell cultures with 70-80% confluency were treated with a different nontoxic concentration of test substances. After 2 hours of treatment, the plate was washed with phosphate buffer saline (PBS). The cellular ATP levels determination was performed according to the instructions given in the kit manual (ab113849). At the end of the experiment, the optical density was read at 405nm using microplate reader. From the absorbance values, the cellular ATP levels were estimated using kit

protocol and the concentration of cellular ATP levels in treated groups were determined in comparison to the control groups.

vi. Study Design

Table 2: Group, dose and treatment

Sl. No.	Group	Dose and Treatment	Parameters Analysed
1	Cell Control	No treatment	Cellular ATP levels
2	Positive Control	Cells were treated with H_2O_2	
3	**G1** (250 µg/ml)	Cells were treated with test substances	
4	**G2** (250 µg/ml)	Cells were treated with test substances	
5	**G1** (250 µg/ml) + Positive Control	Cells were treated with test substances and H_2O_2	
6	**G2** (250 µg/ml) + Positive Control	Cells were treated with test substances and H_2O_2	
7	**G3** (250 µg/ml)	Cells were treated with test substances	
8	**G3** (250 µg/ml)+ Positive Control	Cells were treated with test substances and H_2O_2	

Table 3: Cytotoxicity properties of test substances against HepG2 cell line.

Sl. No	Name of Test Compound	Test Conc. (µg/ml)	% Inhibition	CTC 50 in µg/ml
1.	G1	1000 5000 250 125 62.5	31.76±1.40 26.86±1.26 18.82±2.27 17.13±1.09 10.43±1.79	>1000
2.	G2	1000 500 250 125 62.5	29.08±1.34 24.71±1.59 20.98±1.49 15.38±2.4 9.15±3.5	>1000
3.	G3	1000 500 250 125 62.5	41.9±3.84 31.41±1.41 24.01±1.66 15.91±1.57 8.80±3.17	>1000

Table 4: Effect of test substances on cellular ATP levels in HepG2 cells.

Sl. No.	Name of the test substances	Test Conc. in µg/ml	OD values (treated)-OD values (Blank)@ 405nm	Cellular ATP Conc. (mM)	Percentage enhancement of cellular ATP levels over control
1.	Cell Control	–	0.0±0.0	0.0±0.0	0.00±0.00
2.	Standard Control (H_2O_2-20 µM)	--	0.0153± 0.002	1.28±0.42	6.56±0.27
3.	G1 (250 µg/ml)	250	0.042±0.001	3.5±0.08	16.06±3.45
4.	G2 (250 µg/ml)	250	0.048±0.01	3.97±0.832	19.97 ± 3.54
5	G1 (250 µg/ml) + Standard Control	250	0.032 ± 0.005	2.96 ± 0.46	13.55±2.48
6	G2 (250 µg/ml) + Standard Control	250	0.043 ± 0.005	3.61± 0.4	18.16±0.47
7	G3 (250 µg/ml)	250	0..073±0.05	6.11±0.46	37.99±3.94
8	G3 (250 µg/ml)+ Standard Control	250	0.08±0.007	6.66±0.59	28.31±2.93

Discussion and Conclusion

The test substances were evaluated for their cytotoxicity with different concentrations ranging from 62.5µg/ml to 1000µg/ml. The CTC_{50} value was found to be more than 1000µg/ml for all the test substances. Hence the test concentrations of 250µg/ml were taken for further studies. The cellular ATP levels for the test substances were found to be 2.69±0.46 and 3.61 ± 0.416 for G1 and G2 at the test concentrations respectively. The percentage enhancement of cellular ATP levels was found to be 13.55±2.48 and 18.16±0.47 for G1 + H_2O_2 and G2 + H_2O_2 respectively. The test concentrations are compared over untreated control and Standard control.

The cellular ATP levels for the test substances were found to be 6.33±0.46, and 4.16 ± 0.26 for G3 and G3 + H2O2 at the test concentrations respectively. The percentage

enhancement of cellular ATP is 37.99 ± 3.94 and 28.31 ± 2.93 for G3 and G3 + H2O2 at the test concentrations respectively.

The results indicated that the test concentrations have increased cellular respiration in HepG2 cells. All the test substances have exhibited an increase in cellular ATP levels over untreated control.

We claim;

1. A novel synergistic nutritional composition for treating metabolic syndrome comprising a therapeutic blend of sirtuins (SIRT1) activator(s) and stabilized 5' adenosine monophosphate-activated protein kinase (AMPK) activator(s) present in the ratio of 1: 1 to 1: 5, along with pharmaceutically acceptable excipients.

2. The novel synergistic nutritional composition as claimed in claim 1, wherein SIRT1 activator is a N1-methylnicotinamide salt in the range of 15% to 30% by wt. of total composition.

3. The novel synergistic nutritional composition as claimed in claim 1, wherein the stabilized AMPK activator is a homogenous premix of oxaloacetic acid and an antioxidant in the ratio of 1:1 to 1:2, preferably 1: 1.45 to 1:1.75.

4. The novel synergistic nutritional composition as claimed in claim 3, wherein the oxaloacetic acid is present in the range of 15 to 35% by wt. of total composition.

5. The novel synergistic nutritional composition as claimed in claim 3, wherein the antioxidant is standardized ascorbic acid present in the range of 25 to 50% by wt. of total composition.

6. The novel synergistic nutritional composition as claimed in any of preceding claims, wherein the N1-methylnicotinamide salt to oxaloacetic acid to standardized ascorbic acid ratio ranges from 1:1:1 to 1:2: 2.

7. The novel synergistic nutritional composition as claimed in claim 6, wherein the standardized ascorbic acid comprises 10-250 mg of ascorbic acid.

8. The novel synergistic nutritional composition as claimed in claim 1, wherein the N1-methylnicotinamide salt is chloride salt.

9. The novel synergistic nutritional composition as claimed in claim 1, wherein the pharmaceutically acceptable excipients are selected from a diluent, a binder, a lubricant, a glidant, an additive and a solvent or mixtures thereof.

10. The novel synergistic nutritional composition as claimed in claim 9, wherein the amount of a diluent ranges from 1-35% by wt of the composition; binder ranges from 0.5- 5% by wt of the composition; lubricant ranges from 0.5 to 5 % by wt of the composition; glidant ranges from 0.5-5 % by wt of the composition; additive ranges from 1-10% by wt of the composition.

11. A novel synergistic nutritional combination of N1-methylnicotinamide chloride and oxaloacetate and standardized ascorbic acid in the ratio of 1: 1.1: 1.6.

12. The novel synergistic nutritional combination as claimed in claim 11, wherein the amount of N1-methylnicotinamide chloride is 26 ± 0.5 %; amount of oxaloacetic acid is 29 ± 0.5% amount of standardized ascorbic acid is 44 ± 0.5 % by wt of blend.

13. The novel synergistic nutritional combination as claimed in claim 11, wherein the amount of N1-methylnicotinamide chloride is 18± 0.5 %; amount of oxaloacetic acid is 20 ± 0.5%, amount of standardized ascorbic acid is 30 ± 0.5 % by wt of total composition.

14. The novel synergistic nutritional composition as claimed in claim 1, wherein the effective dose administration of the composition shows enhanced antioxidant activity in a subject in need thereof.

15. The novel synergistic nutritional composition as claimed in claim 1, wherein the effective dosage administration of the composition shows enhanced ATP activity in a subject in need thereof.

16. The novel synergistic nutritional composition as claimed in claim 1, wherein the effective dosage administration of the composition shows improved cholesterol, glucose level in a subject in need thereof.

17. The novel synergistic nutritional composition as claimed in claim 1, wherein the effective daily oral dosage ranges from 10 mg to 500 mg.

18. The novel synergistic nutritional composition as claimed in claim 1, wherein the effective oral dosage is in the form of tablet or capsule or powder or granules or fine particles.

AMENDED CLAIMS

received by the International Bureau on 22 May 2020 (22.05.2020)

1. A novel synergistic nutritional composition for treating metabolic syndrome comprising a therapeutic exogenous blend of N1-methylnicotinamide chloride and stabilized oxaloacetate along with pharmaceutically acceptable excipients, wherein the N1-methylnicotinamide chloride and the stabilized oxaloacetate are present in the ratio of 1:2 to 1:5.

2. The novel synergistic nutritional composition as claimed in claim 1, wherein the stabilized oxaloacetate is a homogenous premix of crystalline organic oxaloacetic acid and standardized ascorbic acid in the weight ratio of 1:1 to 1:2.

3. The novel synergistic nutritional composition as claimed in claims 1 and 2, wherein the N1-methylnicotinamide chloride to the crystalline organic oxaloacetic acid to the standardized ascorbic acid weight ratio ranges from 1:1:1 to 1:2:2.

4. The novel synergistic nutritional composition as claimed in claim 3, wherein the N1-methylnicotinamide chloride is present in the range of 15% to 30% by weight of total composition.

5. The novel synergistic nutritional composition as claimed in claim 3, wherein the crystalline organic oxaloacetic acid is present in the range of 15 to 35% by weight of total composition.

6. The novel synergistic nutritional composition as claimed in claim 3, wherein the standardized ascorbic acid is present in the range of 25 to 50% by weight of total composition.

7. The novel synergistic nutritional composition as claimed in claim 6, wherein the standardized ascorbic acid comprises 10-250 mg of ascorbic acid.

8 The novel synergistic nutritional composition as claimed in claim 1, wherein the pharmaceutically acceptable excipients are selected from a diluent present in the range of 1-35%; a binder present in the range of 0.5- 5%; a lubricant present in the range of 0.5 to 5 %; a glidant present in the range of 0.5-5 %; an additive present in the range of 1-10%, by weight of the total composition..

9. A novel synergistic nutritional combination of N1-methylnicotinamide chloride and crystalline organic oxaloacetic acid and standardized ascorbic acid in the weight ratio of 1: 1.1: 1.6.

10. The novel synergistic nutritional combination as claimed in claim 9, wherein the amount of N1-methylnicotinamide chloride is 26 ± 0.5 %, the amount of crystalline organic oxaloacetic acid is 29 ± 0.5% and the amount of standardized ascorbic acid is 44 ± 0.5 % by weight of the combination.

11. The novel synergistic nutritional combination as claimed in claim 9, wherein the amount of N1-methylnicotinamide chloride is 18 ± 0.5 %; the amount of oxaloacetic acid is 20 ± 0.5%, the amount of standardized ascorbic acid is 30 ± 0.5 % by weight of total composition.

12 The novel synergistic nutritional composition as claimed in claim 1, wherein an effective oral dosage is in the form of tablet or capsule or powder or granules or fine particles.

13. Use of the novel synergistic nutritional composition as claimed in claim 1, for treating metabolic syndrome related to obesity, weight gain, hyperlipidemia, coronary heart diseases, diabetes, hepatic and renal disorders.

14. The novel synergistic nutritional composition as claimed in claim 1, wherein the effective unit dose for oral administration is in the range of 10 mg to 500 mg.

STATEMENT UNDER ARTICLE 19 (1)

Basis of amendment is as follows:

Original Claims 1-6, 9, 11-13, 17 have been amended; Original claims 7 and 18 remain unchanged; Claims 8, 10, 14-16 have been deleted

Original Claim 1 has been amended to incorporate the subject matter of original Claims 2, 3 and claim 8. In particular, claim 1 has been amended to recite "therapeutic exogenous blend of N1-methylnicotinamide chloride and stabilized oxaloacetate along with pharmaceutically acceptable excipients, wherein the N1-methylnicotinamide chloride and the stabilized oxaloacetate are present in the weight ratio of 1:2 to 1:5." Support for the amendment made to original claim 1 can be found in original claim 2, 3 and 8.

Original claim 2 has been amended for consistency and renumbered as claim 4.

Original claim 3 has been amended for consistency and renumbered as claim 2.

Original claim 4 has been amended for consistency and renumbered as claim 5.

Original claim 5 has been amended for consistency and renumbered as claim 6.

Original claim 6 has been amended for consistency and clarification and renumbered as claim 3.

Original claim 7 remains unchanged.

Original claim 8 has been deleted.

Original claim 9 has been renumbered as claim 8 and has been merged to incorporate the subject matter of claim 10.

Original claim 10 has been merged with original claim 9 and has been deleted.

Original claim 11 has been amended for consistency and renumbered as claim 9.

Original claim 12 has been amended for consistency and renumbered as claim 10.

Original claim 13 has been amended for consistency and renumbered as claim 11.

Original claims 14-16 have been deleted.

Original claim 17 has been amended for consistency and renumbered as claim 14.

Original claim 18 has been renumbered as claim 12.

New claim 13 has been introduced which finds support in lines 18 – 23 on page 19 and lines 3 – 8 on page 26 of the specification.

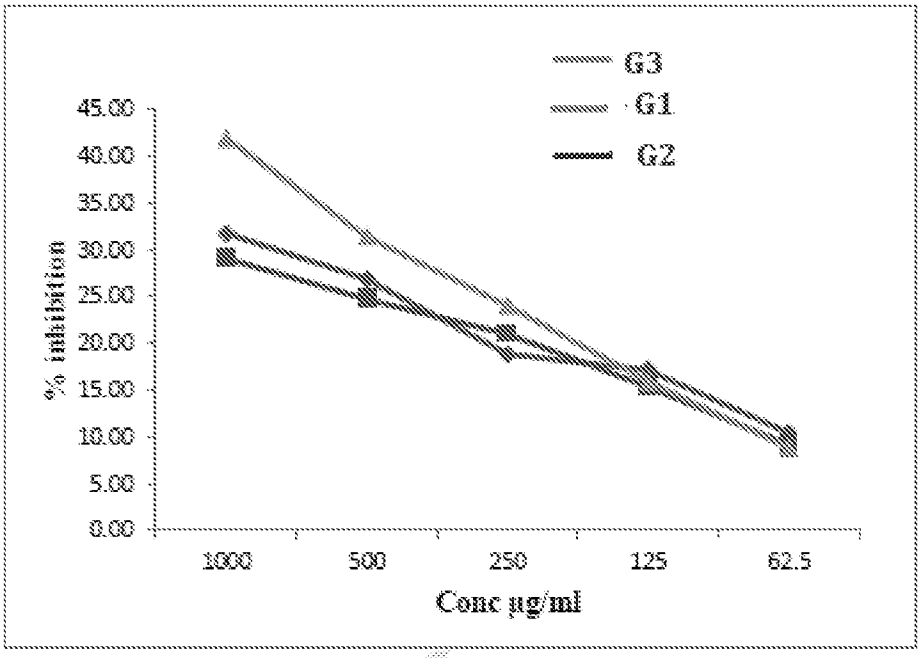
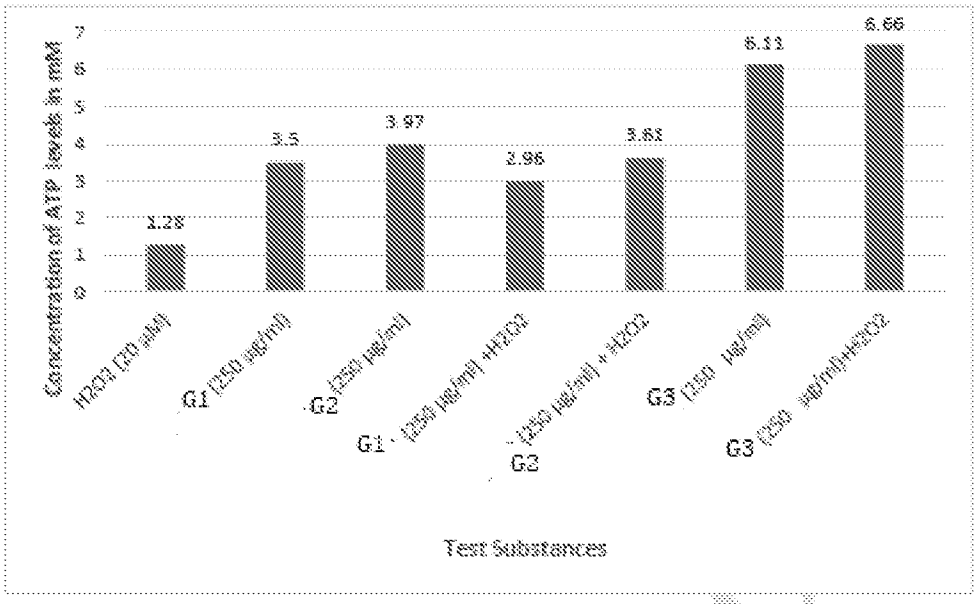

G3
G1
G2
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
% inhibition
1000
500
250
125
62.5
Conc µg/ml

Figure-1


Concentration of ATP levels in mM
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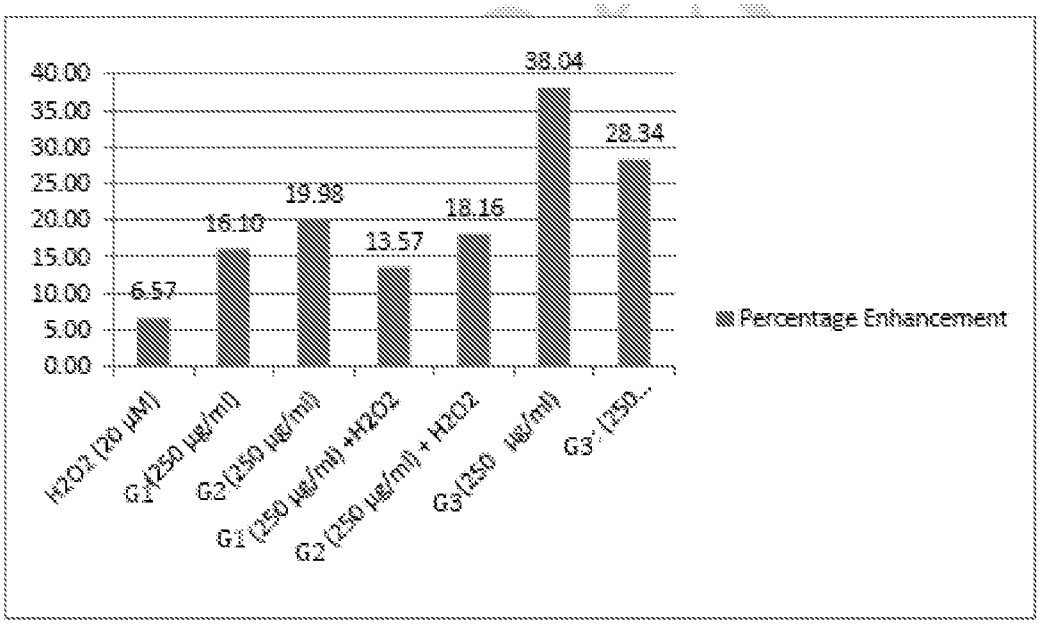
1
2
3
4
5
6
7
1.28
3.5
3.97
2.96
3.61
6.11
6.66
H2O2 (50 mM)
G1 (250 µg/ml)
G2 (250 µg/ml)
G1 + (250 µg/ml) +H2O2
G2 (250 µg/ml) + H2O2
G3 (250 µg/ml)
G3 (250 µg/ml)+H2O2
Test Substances

Fig.2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35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
6.57
16.10
19.98
13.57
18.16
38.04
28.34
H2O2 (20 µM)
G1 (250 µg/ml)
G2 (250 µg/ml)
G1 (250 µg/ml) +H2O2
G2 (250 µg/ml) + H2O2
G3 (250 µg/ml)
G3 + (250...
Percentage Enhancement

Fig.3

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2019/050885

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/20 A61K9/48 A61K31/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, EMBASE, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/149466 A1 (MILBURN MICHAEL [US] ET AL) 28 June 2007 (2007-06-28) paragraph [0005]; example 2	1-18
X	WO 2010/134085 A1 (INST OF LIFE SCIENCES [IN]; KRUTHIVENTI ANIL KUMAR [IN] ET AL.) 25 November 2010 (2010-11-25) page 3; claims 6, 8-10; example 2	1,9, 14-16
A	US 2017/105954 A1 (CASH ALAN B [US]) 20 April 2017 (2017-04-20) paragraphs [0024] - [0026]; claim 19; examples 6-7	1-18
A	WO 2018/015862 A1 (PHARMENA S A [PL]) 25 January 2018 (2018-01-25) claims 1, 9, 20	1-18

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 March 2020	23/03/2020

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IN2019/050885

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2007149466 A1	28-06-2007	NONE	
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		US 2012129923 A1	24-05-2012
		WO 2010134085 A1	25-11-2010
US 2017105954 A1	20-04-2017	US 2013143930 A1	06-06-2013
		US 2015238450 A1	27-08-2015
		US 2017105954 A1	20-04-2017
		US 2019054049 A1	21-02-2019
		WO 2011163319 A2	29-12-2011
WO 2018015862 A1	25-01-2018	NONE	