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Review Article

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Withaferin A – A natural multifaceted therapeutic compound

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ABSTRACT

COVID 19, which has led to the death of millions of people, is still spreading worldwide. Development of any new drug after proper trial is much time consuming. This present global pandemic situation has led the researchers around the world to run behind various existing antiviral and immunomodulatory natural compounds to overcome this contagious disease. *Withania somnifera* (ashwagandha) that is being used in Ayurvedic medicine for several ailments since several years is also said to pocess anti-viral activity. Many of its metabolites are being studied individually for its efficacy against the dreadful disease. Withaferin A, a steroidal lactone compound from ashwagandha is one among the important metabolites which is being investigated for its anti-viral activity. Thus because of its wide spectrum of medicinal properties it has now become an attractive drug candidate for several preclinical studies. This increase in the demand for withaferin A has chanelled its way towards *in vitro* propagation of the plant *W. somnifera* and trials on various strategies to increase the yield in terms of plant biomass as well as the withaferin content in the plants thus making it a better alternative to field grown plants. Thus this article reviews in depth on the important medicinal properties of withaferin A, its demand in Ayurveda industry and the *in vitro* strategies that are being carried out to overcome the demand.

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INTRODUCTION

Withania somnifera Dunal (Ashwagandha), belonging to the family Solanaceae, has been known for its array of therapeutic activities. It is an important medicinal herb in the traditional medicine. Various studies have demonstrated that Withania, in its reasonable dose is non-toxic, safe and an edible herb to be used as an adaptogenic tonic. Until now, 12 alkaloids, 35 withanolides and several sitoindosides have been isolated from this plant and structures elucidated (Mishra *et al*., 2000). Of these withanolides are of prime importance (Matsuda *et al*., 2001). Withanolides, though are a group of naturally occurring steroids specific to the Solanaceae family are also reported in fewer amounts in Lamiaceae, *Taccaceae* and Fabaceae families. It is the major chemical constituent of the *Withania* genus though is reported in small quantities from *Physalis*, *Datura*, *Nicandra*, *Dunalia*, *Lycium*, *Tubocapsicum* and *Jaborosa* genus (Cordero *et al*., 2009). Ashwagandha extracts from various parts like leaf, root and shoots have shown the presence of withanolides, of which withaferin A, withanolide A, withanolide D and withanone are of prime importance.

Withaferin A (WA), a white crystalline substance with maximum absorption in the UV region, is the first member of the withanolides group to be isolated from the leaves of Indian *W. somnifera*. It was then later isolated from an Israeli variety, characterized and given the name Withaferin A (David *et al*., 1965). Being the major pharmacologically active withanolide, it is the most studied molecule (Choudhary *et al*., 2010). WA with potent anti-viral and immunomodulatory activity has also been proved as a potent inhibitor of SARS-CoV2 main protease (Chandel *et al*., 2020) thus becoming an important molecule under focus in this pandemic situation. This review highlights the various studies undergone on this wonder compound thus highlighting the deficiencies in our knowledge. This would further help researchers to direct studies to bridge the gaps.

STRUCTURE AND DERIVATIVES OF WITHAFERIN A IN RELATION TO ITS ACTIVITY

Withanolides are naturally occurring C28 steroids with an ergostane backbone in which the 22nd and 26th carbon are oxidized to form a 6 membered lactone ring. Withaferin A (4β, 27-dihydroxy-1-oxo-5β, 6β-epoxywitha-2-24-dienolide) is a bioactive compound classified as withanolide. Being one of the most bioactive compound, it is necessary to have a clear

insight into the molecular mechanisms underlying its broad range of bioactivities. Therefore, a lot of effort has been made to explore the intracellular effects of WA and to characterize its target protein (Vanden Berghe *et al*., 2012). The structure of WA also contributes to its cytotoxic activity. WA is a C5, C6 epoxy compound with hydroxyl groups on C4 and C27. The skeletal nature of WA was obtained after several dehydrogenation experiments with selenium on the product obtained by lithium aluminium hydride reduction of dihydro withaferin A (Lavie *et al*., 1965a, Lavie *et al*., 1965b). The 2D structure of withaferin A is retrieved from pubchem and presented as figure 1 (https://pubchem.ncbi.nlm.nih.gov/compound/Withaferin-A-*Withania somnifera*). The studies on WA and 9 of its derivatives revealed 4-dehydro withaferin A and withaferin A diacetate as the most potent agents exhibiting equal inhibitory effect on thymidine, uridine, and L-valine incorporation in nucleic acid synthesis. Further the double bond between C2-3 was responsible for the cytotoxic effect, the dissociation of which lead to a decrease in the activity of all the derivatives. Also an increase in the activity was observed on the addition of a carbonyl group at C4 position. Whereas the removal of OH group at C27 or the dissociation of the double bond between C24-25 did not cause any change in the activity (Fuska *et al*., 1984). But, a modification at the OH group of C27 by a subsequent reversible addition fragmentation chain transfer (RAFT) polymerization with hydrophilic N,N-dimethyl acrylamide yielded a completely water soluble conjugate of WA. Thus overcoming its poor solubility that limits its use (Van Herck *et al*., 2019). Similar studies on the structure activity relationships of withaferin A, withanolide D and its semisynthetic analogues prepared by chemical and microbial transformations revealed the increased efficiency of withaferin A derivatives compared to its corresponding withanolide D derivatives.

The study on the cytotoxicity and heat shock inducing activity revealed the importance of the ring A structure for its bioactivities. This study proved that the modification of the withanolide scaffold can increase its heat shock inducing activity (Wijeratne *et al*., 2014). Study on the *in vitro* cytotoxic effect of WA analogues obtained upon modification of the ring A structure reported a 35 fold increase in activity in a 3-azido analogue of WA compared to the parent molecule (Yousuf *et al*., 2011). Metabolite studies on *W. somnifera* grown under soil free aeroponic condition resulted in the isolation two new WA derivatives 3α-(uracil-1-yl)-2,3-dihydroWA and 3β-(adenin-9-yl)-2,3-dihydroWA along with ten other structurally diverse withanolides which were also referred to as WA. The structure of these compounds was elucidated on the basis of high resolution mass and NMR spectroscopy (Xu *et al*., 2011).

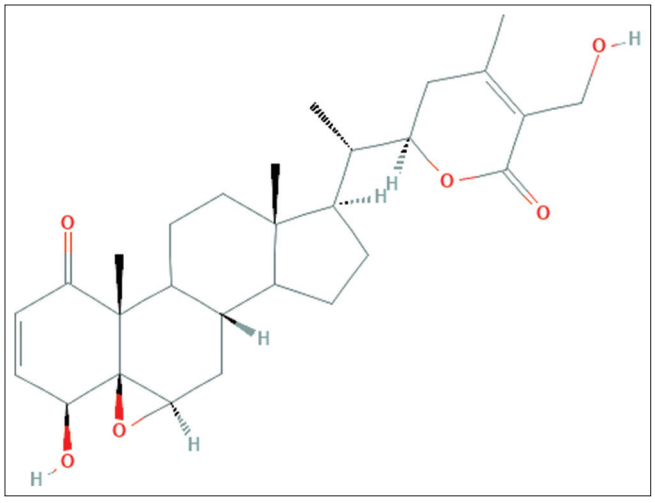

Figure 1: 2D structure of Withaferin A

2,3- dihydro- 3 β-methoxy WA (3βmWi-A), another derivative of WA was found to lack the cytotoxic property of WA but it helped in the induction of anti-stress and pro survival signaling. Thus 3βmWi-A was found to protect normal cells against stress (Chaudhary *et al*., 2019). A few chlorinated forms of WA like 6*α*-chloro-5*β*-hydroxy WA, (22*R*)-5*β*-formyl-6*β*,27-dihydroxy-1-oxo-4- norwith-24-enolide, 2,3-dihydrowithaferin were also isolated from ethyl acetate fraction of *W. somnifera* and their structures elucidated by crystallographic studies (Tong *et al*., 2011).

THERAPEUTIC POTENTIAL OF WITHAFERIN A

Ashwagandha is a cost effective adaptogenic drug that is being used effectively to combat the various complications of stress. Since stress is an underlying cause of most of the health issues, study on the natural ways to overcome it has become a necessity. WA has been reported to increase the antioxidant potential, prevent gastric ulcer and hepatotoxicity induced by stress. Many investigations has been carried out to explore the multifaceted properties of WA and the results showed its significant pharmacological properties (Shrilata *et al*., 2017). The ADME studies using QikProp software revealed that WA is a small molecule capable of exhibiting antagonistic and agonistic activities without any side effects to the organism (Vaishnavi *et al*., 2012). Further the oral bioavailability of WA studied in male rats (Dai *et al*., 2019) showed bioavailability of around 32.4% with a strong first pass metabolism. The most important medicinal properties studied so far are explained below and a few less reported studies are presented in table 1.

Anti-oxidant and Anti-inflammatory Activities

The studies by Mandal *et al* (2010) has proved the pharmacognostic and free radical scavenging efficiency of the different parts of the plant *W. somnifera*. The active principles of *Withania somnifera* (sitoindosides VII-X and WA) showed anti-oxidant activity comparable to the standard drug deprenyl (Bhattacharya *et al*., 1997). The glycowithanolides of *Withania somnifera* was also found to reverse the effects of chronic stress. The oral administration of glycowithanolides 1hour before the induction of foot shock stress in rats bought back the SOD and LPO activities to normal (Bhattacharya *et al*., 2001). WA has proved to reduce oxidative stress against DEN induced hepatocellular carcinoma in rats (Murugan *et al*., 2015). A comparative decrease in the levels of reactive oxygen species was observed in WA treated rats.

The anti-inflammatory activity of *W. somnifera* is attributed to the presence of its major biologically active steroid, WA (Patel

Table 1: Therapeutic properties of Withaferin A

S. No.	Reported activity	Model organism
1.	Analgesic	Abdominal constricted mice (Sabina *et al.*, 2009)
2.	Anti-pyretic	Yeast induced pyrexia mice (Sabina *et al.*, 2009)
3.	Ulcerogenic effect	Gastric ulceration mice (Sabina *et al.*, 2009)
4.	Anti-platelet, anti-coagulant	TNF α activated HUVEC cells (Ku & Bae, 2014a)
5.	Endothelial protein C receptor shedding	TNF α activated HUVEC cells (Ku *et al.*, 2014b)
6.	Amyotrophic lateral sclerosis	$SOD1^{G93A}$ mouse model (Dutta *et al.*, 2018)
7.	Advanced osteosarcoma	Human patients with osteosarcoma (Pires *et al.*, 2019)
8.	Prostrate cancer	PC3 cell lines (Setty Balakrishnan *et al.*, 2017)
9.	Arthritis	Mice (Sabina *et al.*, 2008)
10.	Osteoporosis	Mice (Khedgikar *et al.*, 2013)
11.	Diuretic	Laboratory animals (Benjumea *et al.*, 2009)
12.	Thyroid cancer	DRO81-1 xenograft mice (Samadi *et al.*, 2010)
13.	Obesity	High fat diet induced obese mice *(Dutta et al.,* 2019)
14.	Renoprotective	Male swiss albino mice (Peddakkulappagari *et al.*, 2019)
15.	Cardioprotective	Male wild mice (Guo *et al.*, 2019)
16.	Lung cancer	Cancer cell lines (Hsu *et al.*, 2019)
17.	Alcohol abstinence	Rats (Kotagale *et al.*, 2018)

et al., 2015a). In raw 264.7 cells, WA was found to inhibit LPS induced expression of both nitric oxide synthase (iNOS) protein and its mRNA. Oh *et al* (Oh *et al*., 2008) examined the mechanism by which WA inhibits iNOS gene expression, and suggested that WA inhibited inflammation through inhibition of NO production and iNOS expression, by blocking Akt and subsequently downregulating NFκB activity.

Anti- microbial Activity

The aqueous and organic extracts of the leaf, stem and root powders of *W. somnifera* are reported with potent inhibitory activity against the fungal species *Fusarium oxysporum* and *Radicis lycopersici* (Nefzi & Ben Abdallah, 2016). Kumari & Gupta (Kumari & Gupta, 2015) has reported the inhibitory effect of *W. somnifera* root extract towards *E. coli* showing maximum up to 57.40% inhibition. The inhibitory activity of WA is due to its reaction with the SH group of the enzymes and metabolites required by the organisms. Further it was observed that this activity of WA is blocked by the presence of glutathione in the media (Budhiraja *et al.*, 2000).

Human papilloma virus (HPV), the cause of cervical cancer, has been reported to be the main cause of cancer death in women worldwide. The high risk HPV 18 was found to inactivate the p53 (tumor suppressor) protein by its interaction through E6 oncoprotein. Since WA has been used traditionally for the cure of various cancers, its interaction with the oncoprotein was studied. The docking results revealed that WA interacts with the residues 108-117 of the p53 binding site on E6 oncoprotein, thus reversing to the normal functioning of p53 (Kumar *et al*., 2015). Another simulation study exhibited the binding potential of the terminal hydroxyl groups of WA to sites on DNA polymerase of Herpes simplex virus. WA was predicted to bind to the amino acid residues Gln 617, Gln 618, Asn 815and Tyr 818 which are essential for the functioning of the enzyme. Thus WA can be used as a potent anti-herpetic drug (Grover *et al.*, 2011a).

COVID 19 is a transmissible severe acute respiratory syndrome caused by SARS-CoV-2 virus. Various anti-viral compounds and active phytoconstituents approved by FDA (Chandel *et al*., 2020) and nearly forty chemical constituents of *W. somnifera* were studied using molecular dynamic simulation for its activity against main protease protein of SARS-CoV-2 (Tripathi *et al*., 2020). Withaferin A displayed a strong interaction towards the main protease, spike glycoprotein and RNA dependent RNA polymerase of SARS-CoV-2, with a binding energy of -11.242kcal/mol, -9.631 kcal/mol and -9.27 kcal/mol respectively (Pandit, 2020). Withaferin A was shown to interact with eleven residues Thr 24,Thr25,His 41,Cys 44,Ser 46,Met 49,Phe 140,Leu 141,Asn 142,His 164 and Glu 166 of main protease Mpro with residues(Sharma & Deep, 2020). Withaferin A of *W. somnifera* has also been reported to target and repress TMPRSS2 protein which acts as a gateway for entry of viruses into host cells (Wadhwa *et al.*, 2020) and the cellular receptor GRP78 protein which facilitates viral entry into cells (Sudeep, 2020). The severity of this infectious disease was found to be increased in aged people and people with cancer or other comorbidities. Administration of withaferin A was found to bind the viral protein and thereby inhibiting host ACE2 receptor, the downregulation of which leads to an increase in the spread of the disease (Straughn, 2020).

Leishmaniasis, an endemic disease caused by protozoan leishmania has now become an area of concern since a few species like *L. donovani* has developed resistance to the available drugs. Thus making it difficult to cure and therefore there is an urge to discover new drugs that can target different sites of the protozoan and thus cure the disease. Molecular dynamic studies has revealed the inhibitory activity of WA on leishmanial protein kinase C, the target protein for anti- leishmanial drugs (Grover *et al*., 2012). Another important enzyme that can be targeted for developing an effective anti-leishmanial drug is Pteridine reductase 1(PTR1). The molecular docking studies of WA against PTR 1 showed the binding efficacy with lowest binding energy of -6.73kJ/mol (Chandrasekaran *et al*., 2015). Due to structural similarity between the PTR1 and DHFR-TS, the inhibitor that targets PTR1 can also target DHFR-TS. Thus the molecular dynamic studies by (Vadloori *et al*., 2018) against Dihydrofolate reductase- thymidylate synthase (DHFR- TS) revealed two binding sites of WA one on DHFR and another on TS domain both 40Å apart.

Anti -diabetic Activity

Withaferin was found to combat the palmitic acid induced oxidative stress effect and thus increase the viability of HUVEC cells in a dose dependent manner. This suppression of oxidative

stress and inflammation protects the cells against endothelial insulin resistance (Batumalaie *et al*., 2016). In the presence of Insulin, exposure to palmitic acid generally inhibited insulin mediated IRS-1 tyrosine phosphorylation, which leads to the deterioration of downstream insulin. This inhibitory effect was mitigated by the pre-treatment of the cells with WA. Jonathan (Jonathan *et al*., 2015) reported the increased efficiency of WA for the uptake of glucose by the skeletal myotubes among the six withanolides studied. Thus, proving its role in the anti-diabetic activity of the plant. Further he also reported the increased efficiency of the leaf extracts for the uptake of glucose than the root extracts. The role of WA was further revealed by the increased uptake of glucose by the tissues elicitated with methyl salicylate and chitosan (75% and 69% increased WA) than non-elicitated plants.

Hepatoprotective Activity

W. somnifera root extracts showed protective role by reversing the liver marker enzymes to normal in bromobenzene induced oxidative stress model (Vedi *et al*., 2014). WA has been reported to prevent as well as therapeutically cure liver injury thus lowering liver inflammation and fibrosis in mice with non-alcoholic steatohepatitis (Patel *et al*., 2019). Jadeja *et al*, (2018) in his study on the effect of WA (40mg/kg) on acetaminophen induced liver injury in mice, reported reduced hepatocytic necrosis and intrahepatic hemorrhage. The treated mice also showed reduced JNK (c-Jun N-terminal kinase pathway) activation, mitochondrial Bax (BCL2 associated X protein) translocation and nitrotyrosine generation all of which are induced by acetaminophen in untreated mice. In addition to NFkB activation and vimentin inhibition, the role of WA in altering the expression of LOXL2/ Snail1 and thus reversing liver fibrosis has also been studied (Sayed *et al*., 2019). WA was also found effective in the inhibition of hepatocellular carcinoma via growth inhibition and autophagy (Siddharth *et al*., 2019). WA, being cytotoxic compound is also toxic to normal cells. So in order to selectively chemosensitize the cancer cells to WA, a new strategy of fortifying the crude extracts of Ashwagandha with WA was found to give better results (Sharma *et al*., 2011).

Neuroprotective Activity

The abnormal accumulation of amyloid β is the main reason underlying the pathogenesis in neurodegenerative diseases like alzheimer. This abnormal secretion of amyloid β is induced by a neurotoxic protein Tat and drug cocaine. Studies on SH-APP (Amyloid precursor protein –APP plasmid transfected SY5Y cells) cells revealed that WA is an potent inhibitor of Tat and cocaine induced amyloid β levels and preventing neurotoxicity (Tiwari *et al*., 2018). The MTT assays on the Human neuroblastoma (IMR32) and Rat glioblastoma (C6) treated with alcoholic and water extracts of leaves has showed an increase in the viability of the cells by 20-30% compared to untreated controls. The oxidative and DNA damage stress caused by H_2O_2 were also recovered in leaf extract treated cells (Shah *et al*., 2015). Withaferin A also showed a graudual increase in the expression levels of the neurotropic genes BDNF (Brain derived neurotrophic factor) and GDNF (Glial cell line derived neurotrophic factor) and a suppression in the mRNA expression of the neurite outgrowth inhibitory genes Nogo-a and Rho A compared to untreated SCI mice. Further WA in addition to down regulation of the pro- apoptotic protein bax also upregulates the anti- apoptotic protein bcl-2 thus reducing the apoptotic cells which would otherwise be enhanced in SCI induced mice models. Thus reporting a obvious difference in the neurobehavior in Spinal cord injury (SCI) model mice treated with WA. The administration of WA also attenuated the inflammatory response by suppressing the production of IL-1b and TNF-a (Yan *et al*., 2017).

Ashwamax, a commercially available root extract of *W. somnifera* was tracked orally using bioluminescence imaging in orthotopic murine models with glioblastoma. WA is reported to effectively inhibit the growth of glioma cell lines (Chang *et al*., 2016). The effect of WA on neuroglial cells was studied in vitro using C6 glioma cells and the IC50 value was reported as 355 μg/ml, 230 μg/ml and 150 μg/ml for 24hr, 48hr and 72hr respectively. Further MTT assay deduced a significant reduction in C6 glioma cell count upon WA treatment thus proving its anti-proliferating capability in a time dependent manner. Increased DNA fragmentation was also observed in C6 cells along with increase in WA concentration with maximum fragmentation at 500 μg/ml. Further analysis on normal and C6 cells revealed that WA triggers apoptosis in cancer cells only and not in healthy cells (Hou *et al*., 2017)

Chang *et al* (2017) reported the synergistic inhibition of Glial cell proliferation by WA and Tumor treating fields (TTF). TTF is a novel approach which forces the actin filaments to align along the electric field lines thus interrupting the functionality of mitotic spindle in dividing cells. A study by Chang *et al*. (2016) provides a statistical evidence of reduction in Glioblastoma by 55% when WA was used in combination with TTFs which showed 25% recovery when used alone.

Anti- cancer

Cancer, being a formidable health challenge is the second leading cause of mortality. Though a wide variety of anti-cancer drugs have been developed, the global incidence of various cancers, and the mortality thereof, is still increasing. The number of cancer deaths is asssumed to increase by two-fold in the next 50 years (Mann *et al*., 2005). Since the growth and development of tumors are triggered by oxidative stress and chronic inflammation, phytochemicals with anti-oxidative and anti-inflammatory properties are thought to play important roles in the prevention and/or treatment of cancer. Different parts of the medicinal plant *W. somnifera* are used in a variety of traditional ayurvedic formulations. As per research, daily administration of Withania root reduced proliferation of tumour cells in methyl nitrosourea-induced (similar to estrogen receptor positive cancer model) rat mammary tumorigenesis (Khazal *et al*., 2013). These anticancer properties of withania are attributable to withanolides, a class of bioactive constituents isolated from *W. somnifera*. Several withanolide compounds isolated from various *Withania* species have shown antitumour activity (Mathur *et al*., 2006). *W. somnifera* hydroalcoholic

extracts have reduced the tumour incidence and its volume in methylcholanthrene induced fibrosarcoma in mice. The treated mice also showed significant changes in the liver biochemical parameters compared to the control mice (Prakash *et al*., 2001). WA is an effective anticancer molecule widely used for the treatment of wide range of cancers. It works by inducing apoptosis in cancerous cells via the generation of ROS and suppression of Bcl-2 (Mayola *et al*., 2011). Since the last two decades, WA is being reported to poses tremendous cytotoxic activity suggesting its potential as an anti-cancer agent (Dutta *et al*., 2019). The docking efficiency of WA against four target proteins mortalin, p 53, p 21 and Nrf2 were studied by Vaishnavi *et al.* (2012) who reported that WA binds strongly to these target proteins and hence is considered as a strong cytotoxic agent. The *in silico* studies for understanding the molecular insights into the process of senescence induced by WA was reported by Bhargava *et al.* (2019). This study revealed the nuclear translocation of Nuclear factor kappa B (NFκB) and a Mitogen activated protein kinase (p38 MAPK) activation selectively in the cancer cells. This NFκB activation underlays various chronic ailments. The molecular mechanism behind the suppression of NFκB activation by WA has been elucidated using molecular dynamics simulation studies. The formation of NEMO- IKKβ complex is an essential step towards NFκB activation and its signaling pathway. The docking studies on WA revealed its potential to interact with the NFκB essential modulator (NEMO) chains to form a stearic barrier that prevents the binding of the upcoming IKKβ. WA was also found to actively distrupt the existing NEMO- IKKβ complex, thus suppressing the NFκB activation (Grover *et al*., 2010b). WA was reported with greater binding energy towards active sites of cyclooxygenase(COX-2) than all the other phytoconstituents of *W. somnifera* studied using graphical software (MOLSOFT) (Prabhakaran *et al*., 2012).

Heat shock protein 90 (Hsp 90) is another molecular chaperone compound that serves as a target for the anti-cancer drugs. The docking efficiency of WA to distrupt the Hsp 90-Cdc37 (chaperone- cochaperone complex) supports the anti-cancer activity of WA(Grover *et al*., 2011b). The Structure Activity Relationship (SAR) study of WA exposed that the epoxy group at C5(6) was responsible for the binding of withanolide to Hsp 90 and inhibit its chaperone activity. Further the hydroxyl group at C4 of ring A enhances the inhibition activity on Hsp 90. It also distrupts its interaction with Cdc37 (Hsp 90- Cdc 37) thus enhancing the anti-proliferative activity of WA (Gu *et al*., 2014). The SAR and ADMET(Absorption, Distribution, Metabolism, Excretion and Toxicity) studies on WA incorporated with silicon in addition to carbonyl group at C4 was found to enhance the activity on human epithelial ovarian carcinoma thus proving it as an essential candidate in ovarian cancer studies (Perestelo *et al*., 2019).The anti-tumour and radiosensitizing effects of WA was studied in Ehrlich ascites carcinoma in swiss mice and reported to be effective against tumour with a LD 50 value of approximately 80mg/ kg (Sharada *et al*., 1996). The effect of WA on several malignant cell lines has been assessed and it has proved to have anti-proliferative effect against acute lymphoblastic leukemia (ALL) and human myelogenous leukemia. Mandal and his coworkers (Mandal *et al*., 2008) explored the effect of WA on p38MAPK signaling pathways in apoptosis of leukemic cell lines and primary cells of patients with ALL.

Falkenberg *et al*., (2017) used Myb reporter cell line HD11-C3-GFP1 (chicken myeloid cell line with eGFP reporter gene) to screen for small molecules that inhibit Myb- dependent gene expression. His study proved withaferin A as a potent inhibitor of Myb- dependent transcription factors thus inhibiting tumorigenesis. Analysis of the mechanism of inhibition showed WA as a more potent inhibitor of C/EBPβ (a transcription factor cooperating with Myb in myeloid cells) than Myb by distrupting the interaction of C/EBPβ with its coactivator p300. Thus proving C/EBPβ as a novel target for WA action. Further molecular dynamics simulation and docking studies have proved WA as an mammalian proteosome inhibitor (Grover *et al*., 2010a).

Angiogenesis, the growth of new blood vessels from pre-existing vasculature, occurs in a variety of unrelated pathological conditions, such as the growth of solid tumors (Folkman *et al*., 1971). Vascular endothelial growth factor (VEGF) is a key signal protein that regulates angiogenesis. But the over expression of VEGF can lead to cancer. The available anti-VEGF drugs pose severe side effects in humans. Hence there is a need for some natural compound that targets VEGF. The docking studies of WA with VEGF showed favourable results highly comparable to the commercial drug Bevacizumab (Saha *et al*., 2013). NF-kB is a well-known transcription factor that controls gene expression of a variety of angiogenesis-related molecules (Klein *et al*., 2002) and mediates oxidative stress-dependent endothelial cell tube formation in collagen-I gels (Shono *et al*., 1996). The subsequent chloroform enriched fraction obtained from the methanolic extracts of *Withania* was found to pocess NF-kB inhibitory activity at an IC 50 value of 12nM. Further the HPLC fractionation of the chloroform extract showed the WA peaks which are responsible for the inhibitory activity.

WA also showed a dose dependent activity on the expression of cyclin D1, a key cell cycle regulator with an IC_{50} value of 112nM. In the endothelial cell sprouting assay on HUVEC (Human Umbilical Vein Endothelial Cell) cells, WA was found to inhibit cell sprouting at doses similar to that of NF-kB inhibition. It was also reported that the inhibition of NF-kB in HUVEC occurs via interference with the ubiquitin-mediated proteosome pathway. Thus WA was found to be an potent anti-angiogenic factor at doses 500 fold lower than that of its reported anti-tumour activity (Mohan *et al*., 2004). WA also binds to vimentin (intermediary filament protein) thus inhibiting capillary growth in corneal neovascularized mouse model (Bargagna-mohan *et al*., 2011).

WA has the capability to upregulate the prosurvival pathway thus effectively treating metastatic diseases. It has shown a 100% reduction in tumor growth in Uveal melanoma malignancy (Samadi *et al*., 2012). WA treatment has shown the suppression in growth of mouse melanoma B16F1 cells *in vivo* (Uma Devi *et al*., 2000) and reduce the growth of Ehrlich ascites tumor in swiss albino mice and prolong its survival by inhibiting tumor

growth via alteration in the spindle microtubule formation (Shohat *et al.*, 1976). WA also caused apoptosis in osteocarcoma (OS) cell lines by distruption of mitochondrial membrane potential and generation of reactive oxygen species. This compound also showed a dose-dependent inhibition of OS cell lines by triggering the caspase-3 activation in U2OS cells in a time-dependent manner with IC_{50} ranging from 0.32 to 7.6 μM. The western blot analysis revealed that WA induced cytosolic cyt-c, and PARP protein expression in addition to caspase 3 in a time-dependent manner (Li *et al.*, 2017). The study on the effect of pure WA and lyophilized root extracts of *W. somnifera* on the isolated skin melanophores of frog proved its significant melanin dispersal effects in a dose dependent manner (Ali & Meitei, 2011).

Administration of WA (2mg/kg body weight) was found to inhibit the weight and volume of human colon cancer (HCT116) cells xenograft in BALB/c mice at the end of six weeks treatment. This suppression in tumour growth was also accompanied by reduced expression of Proliferating cell nuclear antigen (PCNA) in treated xenograft tumour (Choi & Kim, 2015). Yu *et al.* (2011) also reported the inhibition of pancreatic cancer (Panc-1) cells xenograft tumor growth by induction of intra tumoral cell death by 30% and 58% on administration of WA at 3 and 6 mg/kg body weight, respectively. Further western blot analysis revealed that WA actually targets the heat shock protein Hsp 90 in pancreatic cell lines and inhibited its chaperone activity. Studies by Tiruveedi *et al.* (2018) showed around three fold decrease in serum amylase and lipase (biomarkers in acute pancreatitis) on WA treatment. The anti-cancer potential of WA was also found to be associated with the cell cycle arrest at the G2/M phase along with the expression of apoptotic proteins in human gastric adenocarcinoma (AGS cell lines) (Kim *et al.*, 2017).

Oral cancer is another most frequent major health problem with high mortality rate in developing countries including India. Panjamurthy *et al.* (2009a) studied the protective role of WA towards oral cancer. He evaluated the effect of WA on molecular pathogenesis of oral cancer by immune supression of p53 and bcl2 proteins. Oral administration of WA also prevented the alterations in p53 and bcl-2 protein expressions which are normally observed in carcinoma cells. Panjamurthy *et al.* (2009b) in another study on the same model reported the detoxication of the carcinogens, thus blocking their interaction with the cellular macromolecules.

The role of WA in DMBA(0.5% 7,12-dimethylbenz(a)anthracene) induced oral squamous cell carcinoma in golden Syrian hamsters reported the decrease in Na+/K+ ATPase activity and Sodium level along with increase in potassium level of tumour bearing hamsters which is probably due to increased lipid peroxidation and red cell fragility (Manoharan *et al.*, 2009). Whereas, oral administration of WA was found to restore Na+/K+-ATPase activity , red cell fragility and levels of membrane TBARS thus preventing membrane abnormalities in Oral carcinogenesis.

Breast cancer, the most common form of cancer in women is a heterogenous disorder broadly classified into different types each with a distinct gene expression signature (Perou *et al.*, 2000). Though most of the cancer treatments target the primary tumour, the major cause of death in all the tumour types is metastatic disease. Metastatic cells are difficult to detect, highly aggressive, chemoresistant and experimentally difficult to model. The QSAR and ADMET studies on withanolides have revealed WA and eight other compounds to have a high activity against the Sk-Br-3 cells. The molecular docking studies also showed higher affinity of WA to β-tubulin with a binding score of 5.4153 which is higher than the reference compounds 5-fluorouracil (5-FU) and camptothecin with a docking score of 2.5304 and 4.1837 respectively (Yadav *et al.*, 2017). The binding of WA covalently to the surface of tubulin at Cys303 and its down regulation in MCF cells has also been reported (Antony *et al.*, 2014). The normal mammary cell lines did not respond to WA, whereas, it decreased the viability of both MCF7(estrogen responsive) and MDA-MB-231(estrogen independent)cell lines (Stan *et al.*, 2008). This decrease in viability of the cell lines by WA was through the induction of ROS mediated paraptosis via down regulation of the paraptosis inhibitor Alix/AIP-1 (Hahm *et al.*, 2011). WA also decreased the viability of SUM159 cells and suppressed ATR(Ataxia telangiectasia and Rad3□related protein) thus causing cell arrest at G2/M phase (Hahm *et al.*, 2019). Further the apoptosis caused in cultured breast cancer cell lines on WA treatment was attenuated by knockdown of multi domain proapoptotic protein Bax and Bak. Generally the level of Bak protein was found to be higher in WA treated rat models. The MNU (N-methyl-N-nitrosourea) induced breast cancer rat models showed chemoprevention after WA treatment by a gradual decrease in number of tumours per animal and tumour weight (Samanta *et al.*, 2017). The presence of pharmacological doses of WA was reported to significantly inhibit the viability of human breast cancer cells. WA has also proved to be an potent inhibitor of breast cancer stem cells (bCSC) *in vitro* (Kim & Singh, 2014) and also lowered the levels of Forkhead box Q1 (FoxQ1), one of the protein responsible for bCSC maintenance (Kim *et al.*, 2016).

The recent genome sequencing studies have revealed the occurrence of a point mutation in the estrogen receptors making it resistant to tamoxifen (breast cancer drug). The molecular docking studies of WA proved its effective binding to the estrogen receptors both in the presence and absence of the mutation thus exhibiting it as an natural anti-cancer drug against breast cancer (Ali *et al.*, 2020).

The proteomic study on mouse mammary tumor virus–*neu* (MMTV-*neu*) transgenic model showed the down regulation of various glycolytic proteins on WA treatment (Hahm *et al.*, 2013). Vimentin is a filament protein that actually is expressed in mesenchymal cells and functions in cell motility. Some epithelial cancers and tissue sarcomas that exhibit epithelial to mesenchymal transition express vimentin (Lahat *et al.*, 2010). WA binds directly to vimentin *in vitro* (Bargagna-mohan *et al.*, 2011) further in *in vivo* wounding migration assay, WA treated cells lacked vimentin in contrast to the untreated cells that showed vimentin extending into a polarized, actin-containing lamellipodia. Similarly time lapse imaging of vimentin by transfecting vimentin:GFP into MDA-MB 231 breast cancer

cells and treating with WA at higher doses over shorter periods of time showed perinuclear vimentin bundling at 60 min followed by rapid vimentin depolymerisation beginning in the cellular periphery and moving inwards towards the nucleus and induce apoptosis. Thus proving WA has a vimentin binding region and causes depolymerisation of cellular vimentin in migrating breast cancer cells. Higher doses of WA at 4 mg/kg inhibited both metastasis and tumor growth (Thaiparambil *et al*., 2011).

The radiosensitizing effect of WA on the erythrocyte anti-oxidant in carcinoma of uterine cervix has been reported (Reshma *et al*., 2007). WA was reported to inhibit the growth of human breast cancer cells. WA also accelerated the accumulation of p53 (tumour suppressor protein) (Munagala *et al*., 2011). The WA treatment alone or in combination with cisplatin was found to reduce tumour growth and metastasis up to 80% compared to untreated mice (Kakar *et al*., 2014). Yang reported that the oral administration of WA caused apoptosis in prostrate cancer cells by acting on its target proteosome(β5 subunit) (Yang *et al*., 2007).

BIOSYNTHESIS OF WITHAFERIN A

Withaferin A, being the most actively studied molecule, study of its biosynthetic pathway, the genes involved in it and identifying a better genotype has always been an ultimate goal of researchers to increase its metabolite content (Gupta *et al*., 2011). Withanolides being 30 carbon containing triterpenoids are synthesised by the isoprenoid pathway which includes cytosolic mevalonate (MVA) and plasticidal non mevalonate pathway or methylerythritol phosphate (MEP) pathway (Figure 2) (Sabir *et al*., 2013). Both these pathways participate in the withanolide synthesis leading to the synthesis of isopentenyl phosphate (IPP) (Chaurasiya *et al*., 2012). Farnesyl diphosphate (FPP) which is formed by the head to tail condensation of IPP is the main precursor for all the other triterpenoids (Kuzuyama, 2002).

Most of the genes encoding various enzymes of withanolide pathway have been characterized (Dhar *et al*., 2015). Further the effect of various elicitors on these pathway genes and on metabolite production are being worked on by various groups. The various enzymes involved in withanolide synthesis are tabulated in Table 2.

The expression analysis of these pathway genes reveal their differential expression in different tissues, chemotypes and in response to different elicitors (Agarwal *et al*., 2017). Further the differential expression of these genes at different ontogenic stages and its positive correlation with the withanolide accumulation in *in vitro* shoot and root cultures of *W. somnifera* has also been reported (Sabir *et al*., 2013). Among the genes characterized, the over expression of the key regulatory gene, Squalene synthase(SQS) was found to increase WA content in the leaves by 4–4.5 fold (Patel *et al*., 2015b). Further the virus induced silencing of this gene lead to a drop in withanolide synthesis thus stating its crucial role in the pathway (Singh *et al*., 2015). Similarly the over expression of Cycloartenol synthase (CAS) gene increased the WA content by 1.06 to 1.66 fold (Mishra *et al*., 2016). The FPPS gene also plays an important role in withanolide synthesis and accumulates in response to stress conditions (Gupta *et al*., 2011). Senthil *et al* (2015) has reported a strong positive correlation of Farnesyl diphosphate synthase (FPPS) and Squalene epoxidase(SE) genes towards the accumulation of WA in 45 days and 60 days old leaf cultures.

Among the plant parts, leaves were found to express higher quantities of WA. This differential accumulation of the secondary metabolites in different organs is attributed to its tissue specific regulation of synthetic genes (Pandey *et al*., 2017). Further the radiotracer studies using 24-methylene cholesterol as a precursor reveals the possible transportation of withanolides from leaves to the roots (Sangwan *et al*., 2008). In addition, the transcriptional profiles of five important pathway genes (Squalene synthase, squaleneepoxidase, cycloartenol synthase, cytochrome P450 reductase 1 and cytochrome P450 reductase 2) were found to show a significant difference in the root and shoot tissues which were also parallel to the metabolite accumulation with elevated gene expression and metabolite accumulation in the leaves thus indicating the de novo tissue specific synthesis of withanolides. The sequencing of the leaf and root transcriptome by Gupta *et al.* (2013) has also paved a way towards understanding the tissue specific synthesis of plant secondary metabolites.

This tissue specificity can be altered by altering conditions in the environment. One such factor is the presence of endophytes which has a greater potential for sustainable agriculture. Among the various chitinolytic bacterial endophytes isolated from medicinal plants, *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens*, were found to influence withanolide biosynthesis as well as to render tolerance against *Alternaria alternate*. An upregulation of the pathway genes were also observed in the endophyte treated plants (Mishra *et al*., 2018). Pandey *et al.* (2018) isolated a total of 29 bacterial and 11 fungal endophytes from the leaves, roots and seeds of *W. somnifera* and studied for its efficiency in increasing biomass and metabolite production *in vitro*. It was observed that the nitrogen fixing endophytes associated with the roots actually upregualted the HMGR (MVA pathway), DXR and DXS (MEP pathway) genes in the roots. Whereas, in the leaves, these endophytes had no major effect on the same genes. Thus these endophytes were able to synthesis WA in the roots which were actually absent or present in trace amounts in the control roots. This might be due to the presence of different tissue specific regulatory factors in the leaves and roots. Thus the constitutive expression of the pathway genes are actually being governed by a set of another regulatory genes termed as transcription factors (TF). TF acts as regulators of all cellular and metabolic functions. They compromise nearly 7% of the coding sequence in the plant genome (Mochida *et al*., 2011). Nearly 3532 annotated transcripts of TFs belonging to 90 different families were obtained from the transcriptomic database for *W. somnifera* leaf and root tissues (Tripathi *et al*., 2017). The comparative analysis of these transcripts from *W. somnifera* revealed the absence of homologous representatives for LWD1 and WUSCHEL TF (WDR gene family) in any other Solanaceae plants. The expression profiles of these two TF were also found to be tissue specific. The LWD1 factor was highly expressed in the leaves than that of the root

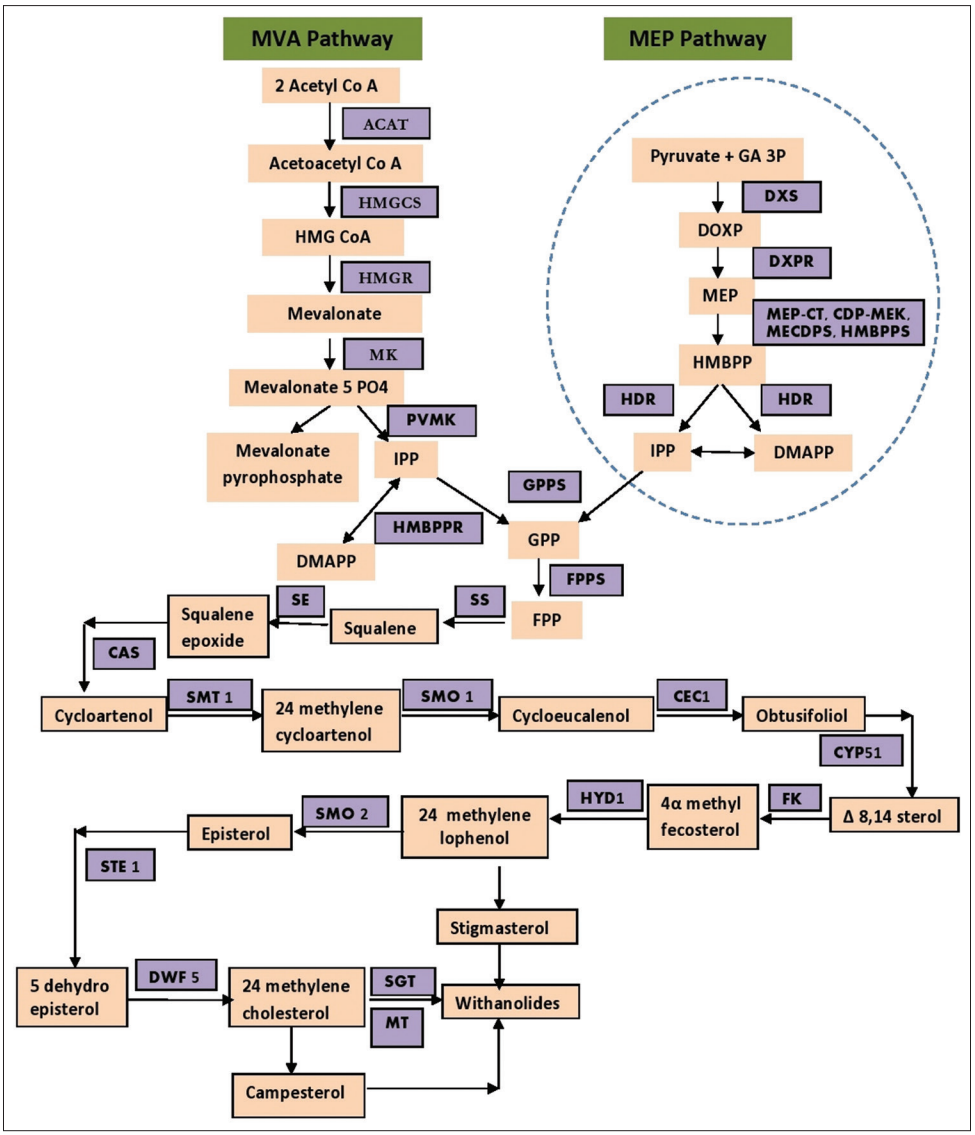

Figure 2: Withanolide Biosynthetic Pathway

whereas WUSCHEL factor was highly expressed in the roots than in the leaves. The over expression of these genes in the transformed tissues was found to be positively correlated to the WA accumulation. Several mi RNA transcriptomes influencing the regulation of withanolide biosynthesis has been identified from the root and leaf tissues of *Withania somnifera*. Out of the 24 and 39 miRNA families identified in the root and leaf tissues, the role of 15 and 27 miRNA families in different biological functions has been found respectively (Srivastava *et al*., 2018).

ROLE OF PLANT TISSUE CULTURE IN WA PRODUCTION

The complexity in the chemical synthesis of withanolides due to the stereo-chemical ring closure, occurrence of chiral centers, rigid trans-lactone groups, and high energy epoxy ring makes it economically unworkable due to minimal yields at high costs. Also the ever increasing demand of bioactive withanolides in the pharmaceutical industry is dependent on field grown plants where the plants are completely uprooted to meet the demands (Mir *et al*., 2014). Further the longer gestation period of 4-5 years between planting and harvesting and the effect on quality and quantity of withanolide constituents by fluxes in genotypic and environmental conditions limits the usage of field grown plants for pharmaceutical industries (Banerjee *et al*., 1994).

Thus, plant cell or organ culture becomes an attractive alternative to enhance the commercial prospects of withanolide production. The mass production in terms of both biomass and WA accumulation can be achieved using appropriate culture conditions when compared to the traditional plant extraction for valuable products (Mir *et al*., 2014). Tissue culture also proves to be a best approach for commercial propagation of endangered species of medicinal plants using various plant growth regulators in different concentrations and combinations (Baskaran *et al*., 2013). In *vitro* cultures are also used as an effective model for the study of production and accumulation of secondary metabolites due to its active growth and increased rate of

Table 2: Role of various enzymes involved in the withanolide biosynthetic pathway

S.No:	Enzyme	Reaction catalysed
1.	Acetoacetyl CoA thiolase (ACAT)	Condensation of two molecules of acetyl-CoA into aceto-acetyl CoA (Wang *et al.*, 2017)
2.	HMG-CoA synthase (HMGCS)	Condensation of acetoacetyl CoA to another molecule of Acetyl CoA (Nagegowda *et al.*, 2004)
3.	HMG-CoA reductase (HMGR)	Double reduction of HMG CoA to mevalonate (Benveniste, 2002)
4.	Mevalonate kinase (MK)	Phosphorylation of mevalonate to produce mevalonate 5-phosphate (Benveniste, 2002)
5.	Phosphomevalonate kinase (PVMK)	phosphorylation of mevalonate 5-phosphate to mevalonate 5-diphosphate (Benveniste, 2002)
6.	1-deoxy-D- xylulose 5-phosphatesynthase (DXS),	Formation of Dxylulose 5phosphate (DXP) (Cordoba *et al.*, 2011)
7.	DXP reductoisomerase (DXPR)	Converts DXP into MEP (Kuzuyama, 2002)
8.	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (MEP-CT)	MEP to 1-hydroxy-2-methyl-2-(E)- butenyl 4-diphosphate (Kuzuyama, 2002)
9.	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CDP-MEK)	MEP to 1-hydroxy-2-methyl-2-(E)- butenyl 4-diphosphate (Kuzuyama, 2002)
10.	2-C- methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECDPS)	MEP to 1-hydroxy-2-methyl-2-(E)- butenyl 4-diphosphate (Kuzuyama, 2002)
11.	(E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HMBPPS).	MEP to 1-hydroxy-2-methyl-2-(E)- butenyl 4-diphosphate (Kuzuyama, 2002)
12.	4-hydroxy-3-methyl but-2-enyl diphosphatereductase (HDR)	Branching of HMBPP to IPP and DMAPP (Kuzuyama, 2002)
13.	Geranyl diphosphate synthase (GPPS)	Conversion of IPP to GPP (Rai *et al.*, 2013)
14.	Farnesyl diphosphate synthase (FPPS)	Head to tail condensation of isopentenyl phosphate (IPP) to farnesyl diphosphate (FPP) (Kim *et al.*, 2018)
15.	Squalene synthase (SS)	Reductive condensation of two molecules of FPP to squalene (Benveniste, 2002)
16.	Squalene epoxidase (SE)	Epoxidation of squalene to 2,3-oxidosqualene (Benveniste, 2002)
17.	Cycloartenol synthase (CAS)	Cyclization of 2, 3-epoxysqualene to cycloartenol (Benveniste, 2002)
18.	Sterol- C24 methyl transferase 1 (SMT1)	Adds methyl group at C-24 position to the sterol cycloartenol (Pal *et al.*, 2019)
19.	Sterol C4 methyl oxidase 1 (SMO 1)	Removal of methyl groups at C4 (Zhang *et al.*, 2016)
20.	Sterol 14alpha-demethylase (CYP51)	Catalyses first cyclization step in sterol biosynthesis (O'Brien *et al.*, 2005)
21.	Δ^7-sterol-C_5-desaturase (STE 1)	Catalyses formation of C-5 double bond in the B ring of Δ7-sterols to yield Δ5,7- sterols (Kamthan *et al.*, 2017)
22.	Glucosyltransferase (GT)	Glycosylation of secondary plant products (Noguchi *et al.*, 2007)
23.	Cytochrome P450 reductase (CPR)	Redox partner of multiple P450s (Bhat *et al.*, 2014)

metabolism in shorter time period (Sivanandhan *et al.*, 2011). Further tissue culture technique could also provide a means to produce disease free healthy plants for drug preparations. It also provides an continuous and controlled homogenous production of metabolites throughout the year (Gawde & Paratkar, 2012).

The WA content in *in vitro* plants was reported to be higher than that of field grown plants. Study reported a 1.14 fold increase in WA content in the *in vitro* leaves of *W. somnifera* compared to the field grown leaves (Sharada *et al.*, 1996). The withanolide content and biomass in the plant were found to be maximum at the exponential phase (7-28 days). The 28 day old plants in suspension were recorded with increased amount of Withanolides (Sivanandhan *et al.*, 2014a). Senthil *et al.* (2015) has reported an maximum WA accumulation (980 ± 0.97 μg/g DW) in 45days old cultured leaves with an gradual decrease over extended period of culture. Similar synchronization with the age of cultures was found in withanolide A accumulation in root cultures.

All the *in vitro* cultured tissues like callus, root and shoot cultures have been investigated for WA production. Ciddi (2006) devised a method for production of WA from cell cultures of *W. somnifera*. The presence of WA in cell cultures was confirmed by TLC analysis and further by HPLC and ES mass spectra. An *Withania* spp, *W. coagulans* root culture was also reported to produce WA of about 11.65 ± 2.30 μg g^{-1} FW (Abouzid *et al.*, 2010). WA was also reported in the *in vitro* flowers (2mg/g DW) and *in vitro* fruits (0.49 mg/g DW) of *W. somnifera* (Sivanandhan *et al.*, 2015). Various workers described various methods for induction of callus tissue of *W. somnifera* and withanolide production from callus tissue. But callus tissues failed to synthesis withanolides, whereas multiple shoot cultures and transformed roots were able to produce withanolides. On the other hand, Chakraborty *et al.* (2013) has stated that the presence or absence of withanolides in any tissue type is dependent on the plant growth regulators used. In his study on callus induction and withanolide quantification using different plant growth regulators, it was surprising that callus obtained from culture media containing 2,4 D and Kinetin showed the absence of withanolide A and withaferin A, whereas the callus from media containing IBA and BAP showed the presence of both these compounds. Thus revealing the role of plant growth regulators on metabolite accumulation. In *in vitro* system, the production of secondary metabolites is dependent upon the media formulations, concentration of sucrose, concentration and type of Plant growth regulators (Ray & Jha, 2001a).

Plant growth regulators are significant factors that influence the growth and metabolite accumulation in plant cell cultures. Any alteration in the concentration of auxin or cytokinin or a change in their ratio would dramatically alter the growth pattern and metabolite accumulation(Rao & Ravishankar, 2002). Among the various concentrations and combinations of plant growth regulators studied (BAP and Kn), MS medium supplemented with 4.44μM BAP was reported with 1.86 fold increase in WA content compared to control (Murugesan. *et al.*, 2017). Similar studies on the effect of plant growth regulators reported that media supplemented with 5.0 μM 6-benzyladenine (BA)

and 1.0 μM Kinetin (Kn) yielded highest amount of WA (13.4±1.15 mg/g of DW). This study also proved that *in vitro* and *ex vitro* shoots contained increased amount of WA in comparison to the field grown shoots whereas the root tissues which contain only a trace amount of WA did not show much variations. Also, a steady increase in WA content was observed from first to fifth week of culture (Mir *et al.*, 2015).

The trials on different concentrations of fertilizers on fresh twigs of *W. somnifera* reported a fivefold increase in WA in twigs treated with DMSO compared to control (Pal *et al.*, 2017). Ammonium sulphate treatment was reported to produce ninety fold increase in WRKY1 transcription factor and an 8 fold increase in WRKY3 transcription factor. The addition of coconut milk (10% v/v) also favoured the increased WA synthesis along with 27 fold increase in the biomass. Supplementation of coconut water along with BAP in liquid media also showed the accumulation of withanolides including WA in micro shoot cultures of *W. somnifera* (Ray & Jha, 2001b). The effect of different carbon sources at different concentrations in enhancing biomass and withanolide accumulation in hairy root cultures of *W. somnifera* were analysed. Among various carbon sources, Sucrose is the most commonly used source because of its capability to translocate easily into the phloem of many plants. They reported a gradual enhancement in the levels of WA along with increase in glucose concentration with the highest accumulation in media containing 5% glucose. Sucrose at 4% exhibited highest WA content but sucrose at 3% equally enhanced both WA and withanolide A accumulation. Fructose at 3% alone showed trace amounts of WA. Thus this work also concludes 3% sucrose to be the best carbon source for *in vitro* cultures of *W. somnifera* (Doma *et al.*, 2012).

The organic additives like L-glutamine (200mg/l) in combination with picloram (1mg/l) and KN (0.5 mg/l)) was reported to show positive effect on biomass and withanolides production (Sivanandhan *et al.*, 2013a). Further withanolide accumulation can be enhanced by the use of elicitors or precursors. Elicitors are factors that can typically cause the cells to activate their defense system through an incompletely understood signal transduction system. Various factors like elicitor concentration, time of incubation, specificity of elicitor, culture conditions and growth stage of cultures influence the process of elicitation (Vasconsuelo & Boland, 2007). Sivanandhan *et al.* (2012a) examined the use of various elicitors (Cadmium chloride, Aluminium chloride and chitosan) and precursors of withanolide synthesis (cholesterol, mevalonic acid and squalene) in suspension cultures using bioreactors and shake flask cultures. Interestingly, a decrease in biomass was observed with the use of chitosan (100mg/L) elicitors with a 1.87 and 1.36 fold higher withanolide content in shake flask culture and bioreactors. Similar decrease in biomass and increased metabolite content upon chitosan treatment was reported by Baldi and Dixit (2008) in A. *annua*. The effect of biotic fungal elicitor (*Piriforma indica*) on biomass and WA accumulation was investigated (Ahlawat *et al.*, 2016). Various concentrations of cell homogenate, culture filterate and culture discs were inoculated into the callus suspension cultures of *W. somnifera* and investigated for biomass production and WA content. A concentration of 3% for cell homogenate, culture filterate and discs were found to enhance WA accumulation at 2.04, 1.78 and 1.46 fold respectively. This elicitation was also reported to produce 11.2, 8.7 and 6.9 fold increased expression of HMG-CoA reductase (HMGR) gene among all the genes studied.

Ciddi (2006) reported the use of methyl jasmonate (100 μM), salacin (750 μM) and arachidonic acid (1 mg/l) as elicitors to improve production of WA. Among these salacin was found to produce 50 fold higher WA content than control. Sivanandhan *et al.* (2013b) also reported a 1.14-1.18 fold increase in WA accumulation in salicylic acid (SA) (100 μM) treated shoots when compared to methyl jasmonate (100 μM) treated shoots in liquid cultures. Further exposure of 30 days old adventitious root cultures for four days with 150 μM SA showed 20 fold increase in its WA content (Sivanandhan *et al.*, 2012b). The sterol inhibitor chlorocholinechloride was reported to inhibit WA production thus proving that precursors enter through the acetate mevalonate pathway rather than through the non mevalonate pathway. Hairy root cultures of *Withania* were also reported to produce WA (Banerjee *et al.*, 1994). The effect of various biotic and abiotic stresses and their combinations on WA production from transformed callus cultures of *W. somnifera* has also been reported (Baldi *et al.*, 2008). Amongst the various elicitors studied, copper sulphate (100μM) and *V. dahaliae* extract (5% v/v) exhibited 5.4 and 9.7 fold increase in the WA production. The combination of an abiotic elicitor Copper sulphate (100 μM) and the cell extract of *V. dahaliae* (5% v/v) as a biotic elicitor showed maximum WA production. The presence of a few new proteins on copper treatment and an increase in the activities of enzymatic antioxidants in the presence of Cu up to 50μM followed by a gradual decrease was observed in cultures (Rout *et al.*, 2013). The combined effect of these two elicitors showed 13.8 fold increases in WA content compared to the control thus proving the potential of dual elicitation strategy for large scale production of WA. Doma *et al.* (2012) also reported the elicitation activity of Chitosan, jasmonic acid, acetyl jasmonic acid and triadimefon on biomass and WA content.

Naturally occurring bioresources such as seaweeds were reported to contain amino acids, vitamins, macro and micro nutrients required for plant growth and also pocess auxin and cytokinin activity. The application of seaweed extracts has been proved beneficial in plant cultivation, improved germination and root development (Mancuso *et al.*, 2006). Hence they also can act as an efficient biotic elicitor. *Gracilaria edulis* and *Sargassum wightii* were studied for its withanolide elicitation property in multiple shoot suspension cultures of *W. somnifera* and reported with 1.45-1.58 fold increase in WA accumulation when compared to control (Sivanandhan *et al.*, 2014b).

A recent study on the effect of Zn-Ag nanoparticles on *in vitro* cultures of *W. somnifera* has shown increase in activity of pathway genes involved in withanolide synthesis and carbohydrate metabolism. Among different molar ratios of Zn and Ag used for nanoparticle synthesis, the nanoparticle synthesised using 19:1 ratio of Zn: Ag showed maximum effect on withanolide content especially WA content in *in vitro* cultures of *W. somnifera* (Singh *et al.*, 2019).

Besides plant growth regulators and elicitors, the variation in physical parameters also affects the plant growth and metabolism. Light is one such very important factor that influences the metabolite production (Adil *et al*., 2019). The study on the effect of different light sources on WA production from callus cultures of *W. somnifera* showed a significant variation in the WA content with different light sources in the order of red> green> Violet> yellow> blue> white. Red light was found to be more favorable for metabolite accumulation with two fold increase in withaferin content compared to tissues grown under white light. A reduction in biomass and increase in lipid peroxidation activity were observed in both leaf and root tissues. Further increased activities of all the enzymatic anti-oxidants were reported in both leaf and root tissues in response to UV stress with greater antioxidant activity in roots (Takshak & Agrawal, 2014).

ISOLATION AND CHARACTERIZATION OF PLANT DERIVED WITHAFERIN A

Whole plants or plant parts are the sole components of Indian ethanomedicine. The bioactive molecules from the plants serve as the basis for synthesis of pharmaceutical drugs. Thus the isolation of active principles has become necessary for which selection of a suitable extraction procedure is required. Study on the effect of ethanol, water and ethanol-water extraction using traditional soxhlet extraction, ultrasound assisted and microwave assisted methods for different time intervals reported a variation in the level of total phenolics and total withanolides as well as DPPH and ABTS activity within different extraction methods and different solvents used (Dhanani *et al*., 2017).

In the case of pharmaceutical compounds, not only the extraction procedure but also the analysis method should be efficient, precise, fast and easy. Though WA is present in abundance in roots and leaves of *Withania*, its amount seems to vary with the geographical niche of the plants. Namdeo *et al.* (2011) has metabolically characterized the leaves, root and stem samples of *W. somnifera* from six different regions of India using H NMR spectroscopy followed by principal component analysis (PCA) and hierarchial clustering analysis (HCA). From the study it was revealed that leaf samples showed wide range of withanolides and the H NMR studies revealed the presence of two groups of withanolides:4-OH and 5,6-epoxy withanolides (WA-like steroids) and 5- OH and 6,7-epoxy withanolides (withanolide A like steroids). He also stated that the proportion of these two withanolides were a key discriminating feature of different geographical locations from where the plants were collected.

A simple yet specific and accurate high performance thin layer chromatographic (HPTLC) method for the estimation of WA using Si 60 F254 plates with Toluene: ethyl acetate: formic acid (5:5:1) as mobile phase has been developed and validated for its repeatability and accuracy (Sharma *et al.*, 2007). This technique is being effectively used in quantification of withanolides. Further the standardization of wavelength for the quantitative scanning of WA was found to be accurate at 223nm in the reflectance- absorbance mode using Scanner III(CAMAG, Switzerland) (Senthil *et al*., 2015). Another new rapid high performance liquid chromatography- mass spectrometry has been developed and validated for the determination of WA from mice plasma (Patil *et al.*, 2013).

WA can be isolated from the root and leaf tissues of *W. somnifera* by various chromatographic techniques. A sensitive, specific, robust, validated densitometric High performance thin layer chromatographic method for the determination of WA from *W. somnifera* has been developed (Srtvastava *et al*., 2008). Keesara & Jat (2017) extracted WA using methanol from the defatted leaf and root powders of *W. somnifera*. The withaferin obtained by this method was tested 90% pure by HPLC analysis. The compound was identified as WA by TLC with chloroform and methanol (9:1 ratio) as the mobile phase. The WA peak obtained was comparable to the standard peak at Rf value 0.65. An modified method HPLC-DAD for the quantification of withanolides including WA and its fingerprinting analysis has been developed and validated (Patil *et al*., 2010).The presence of WA from *W. somnifera* butanol fractions has been identified using Reverse phase preparative HPLC technique (Pramanick *et al*., 2008). An RP-HPLC technique for the isolation and quantification of three isomeric withanolides, WA, withanolide A and withanone using methanol as the mobile phase was developed using Lichrocart Purospher STAR RP-18e column (Malik *et al*., 2017).

CONCLUSION

Various plant derived metabolites are being consumed as a part of our regular diet and others in the form of ayurvedic formulations. Aswagandha being one such important and metabolite rich herb, is an ingredient of most of the ayurvedic preparations for various ailments. Thus, study of its important metabolites and the therapeutic role of each individual metabolite in various ailments would further support the medicinal industry. Further, a study on the measures to improve the yield in terms of metabolite content becomes an necessity. Attempts should be made to scale up the *in vitro* culture process to give maximum output that would be cost effective as well as safe than the traditional methods, thus aiding an improvement in the pharmaceutical industry. This review throws a flashlight onto the most important secondary metabolite WA and would therefore be helpful to the researchers to further explore and innovate methods thus paving a way for industrialization of *W. somnifera*.

The authors declare no conflict of interest.

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