Anti-Inflammatory Implications of Vagus Nerve Stimulation in Colon Carcinogenesis

Thesis

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Submitted By

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(2018)

DECLARATION

I hereby declare that the thesis entitled **"Anti-Inflammatory Implications of Vagus Nerve Stimulation in Colon Carcinogenesis"** has been prepared by me under the supervision of **Dr. Gaurav Kaithwas** at Department of Pharmaceutical Sciences, School for Biosciences and Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow (U.P.).

This work has not been submitted in a part or full to any other University/Institute for any degree/diploma or any other academic award anywhere before. I further declare that the material embodied in the present work is based on original research work and indebtedness to others has been duly acknowledged at relevant places. I hereby also declare that the thesis is essentially free from all kind of plagiarism.

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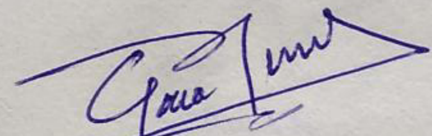

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This is to certify that the thesis titled **"Anti-Inflammatory Implications of Vagus Nerve Stimulation in Colon Carcinogenesis"** submitted by **Mr. Jitendra Kumar Rawat (Enrollment no. 178/11)** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

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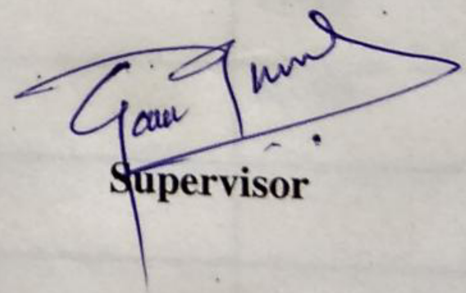

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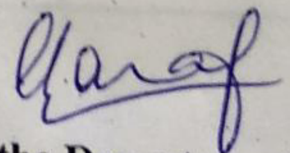

Head of the Department

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ABBREVIATIONS

- CRC- colorectal cancer
- TNF-α -tissue necrosis factor- α
- HPA-hypothalamic pituitary adrenal axis
- DMN-dorsal motor nucleus
- Ach-acetylcholine
- NTS- nucleus of the solitary tract
- ANS- autonomic nervous system
- CAP- cholinergic anti-inflammatory pathway
- CAN-central autonomic network
- α 7nAchR- α7 nicotinic acetylcholine receptor
- LPS-lipopolysaccharide
- nVNS-Non invasive vagus nerve stimulation
- taVNS- transcutaneous auricular vagus nerve stimulation
- PNU-PNU282987
- DMH -1,2-dimethylhydrazine
- HRV- heart rate variability
- HR-heart rate
- ECG- electrocardiogram
- TBAR's- thiobarbituric acid reactive substance
- SOD- superoxide dismutase
- PC- protein carbonyl
- GSH- glutathione
- SEM-scanning electron microscope
- ACF –aberrant crypt foci
- BSA-bovine serum albumin
- DTT-dithiothriol
- TSP -3-trimethylsilyl-(2,2,3,3-d4)-propionic acid
- CPMG- Carr–Purcell–Meiboom–Gill
- BMRB- Biological Magnetic Resonance Data Bank
- PCA- principal component analysis
- PLS-DA- partial least-squares discriminant analysis

- OPLS-DA- orthogonal projection to latent structure with discriminant analysis
- LF-low frequency
- HF-high frequency
- HMBD- human metabolome database
- MMCD- madison metabolomics consortium database
- ROS- reactive oxygen species
- NAG- N-acetyl glycoprotein

CHAPTER 1

INTRODUCTION

Introduction

The condition of abnormal cell division beyond its usual boundaries is called as cancer. Cancerous cells have potential to infect neighbouring tissues and distribute to rest body parts. The self sufficiency in proliferation, unlimited potency of replication, antigrowth insensitivity, elusion for natural cell death, endless vascularisation, invasion and metastasis are some fundamental properties for development of cancer (Hanahan and Weinberg 2000).

Carcinogenesis is the process of development of cancer. On considering worldwide cancer associated death, the colorectal cancer (CRC) is fourth major cause following lung, liver and stomach cancer (Nicholas J Kassebaum, Amelia Bertozzi-Villa, Megan S Coggeshall 2014; Lao and Grady 2011). CRC is third most common cancer (Ferlay J , Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D 2015; Kuipers et al. 2015). The genetic and epigenetic changes accumulated in the colon epithelium, results in development of CRC (Lao and Grady 2011). Various genetic mechanisms responsible for development of CRC, mainly includes chromosomal, microsattelite instability and CpG island methylator phenotype (Tariq and Ghias 2016).

Unlike the other cancer various factors are involve in pathophysiology of CRC, in which inflammation is one of the major reason along with other factors (Mantovani A. 2001). Previous studies established strong genetic interconnection between cancer and inflammation (Mantovani A. 2001; Borrello et al. 2005).

This fact was established a century ago on the basis of observations found in associated studies as development of tumours at chronic inflammatory sites and presence of inflammatory cells in biopsied tumor tissues (Mantovani A. 2001). Several findings from cancer associated epidemiological studies and preclinical

studies performed of genetically modified experimental animals favours the connection of inflammation and cancer.

There are two pathways in relation to inflammation and cancer connection. Both of the pathways are driven by different conditions. Inflammatory conditions drives extrinsic pathway that lead in increased risk of tumour generation. Intrinsic pathway driven by genetic level changes for development of inflammation and cancer(Coussens LM 2002; Balkwill F , Charles KA 2005).

Previous scientific literature reported the involvement of endogenous factors in process of cancer related inflammation. The inflammatory cytokine and transcriptional factors are considered as major endogenous factors. NFκB and STAT3 are considered as major transcriptional factors along with IL-12, IL-23,IL-6 and tumour necrosis factor-α (TNF-α) as major inflammatory cytokines , involved in cancer related inflammation (MKarin 2006).

Various studies reported the immunity linked regulation of inflammation through autonomic nervous system (ANS). ANS and immune system are interlinked in bidirectional way through vagus nerve.

All among the cranial nerves, vagus nerve is longest one (covers from brainstem to abdomen), innervates majority of organ systems including gastrointestinal tract. The vagus nerve originates from medulla in central nervous system and makes a crucial element of ANS (Groves and Brown 2005; Bonaz, Sinniger, and Pellissier 2016). It predominantly reached to the thoracic and abdominal cavity making it different from other cranial nerves. It is having complex course along major arteries and oesophagus, due to this wandering character it gets the name as “vagus nerve”.

Initially vagus nerve was considered as a parasympathetic efferent nerve which controls various autonomic functions (Hr and Wl 2000). After observing

electrocardiogram (ECG) influencing potential of vagus nerve, it is more preferably accepted as mixed nerve (Percival Bailey 1938). Vagus nerve comprises of two types of nerve fibres. Majority of fibres carrying sensory information (efferent, 80%) and rest carrying motor information (afferent, 20%) (Foley and Dubois 1937).

Number wise it is Xth cranial nerve and is also known as pneumogastric nerve. Peripheral and central interface are the most interesting part of vagus nerve since former having specific sensors, effectors and later; with rest of brain. Vagus nerve is involved in controlling immune system along with its classical functions such as controlling heart beat, peristalsis, hormone secretion and digestion (Helke CJ, Goldman W 1980; Goehler LE , Gaykema RP, Nguyen KT, Lee JE, Tilders FJ, Maier SF 1999; R. Ja and Et 1978). It also controls various vital systems via different pathways. Afferent vagus nerve activates hypothalamic pituitary adrenal (HPA) axis and is responsible for anti-inflammatory activity. Efferent vagus nerve exerts immuno-modulatory activity through cholinergic system.

Dorsal motor nucleus (DMN) of vagus nerve is the origin of efferent motor fibre is located in medulla (Ms et al. 2000). Different studies reports different facts regarding to innervations of vagus nerve in gastro-intestinal tract. Efferent vagus nerve fibres do not have direct innervations to lamina propria layer of intestine. Efferent vagus fibres interact with lamina propria layer of intestine through synapses found on to neurons of enteric nervous system and modulate the functions of nicotinic and muscarinic receptors by releasing acetylcholine (Ach) (Berthoud HR , Carlson NR 1991). Some studies reported innervations of vagus nerve throughout the entire gut of humans; whereas in rats, vagus shows innervations similar to human with exception of rectum part of digestive system (Altschuler SM , Escardo J, Lynn RB 1993).

Histologically afferent vagus fibre originates from mucosa to muscular layers of digestive tract. The cell bodies of vagus resides in nodos and jugular ganglia and passes information to the nucleus of the solitary tract (NTS) and area postrema (Goehler, Fleshner, and Watkins 2006; DF 1987). NTS and area postrema forms dorsal vagal complex in close relation with DMN of vagus nerve. Dorsal vagal complex shows its involvement in autonomic, limbic and endocrine responses.

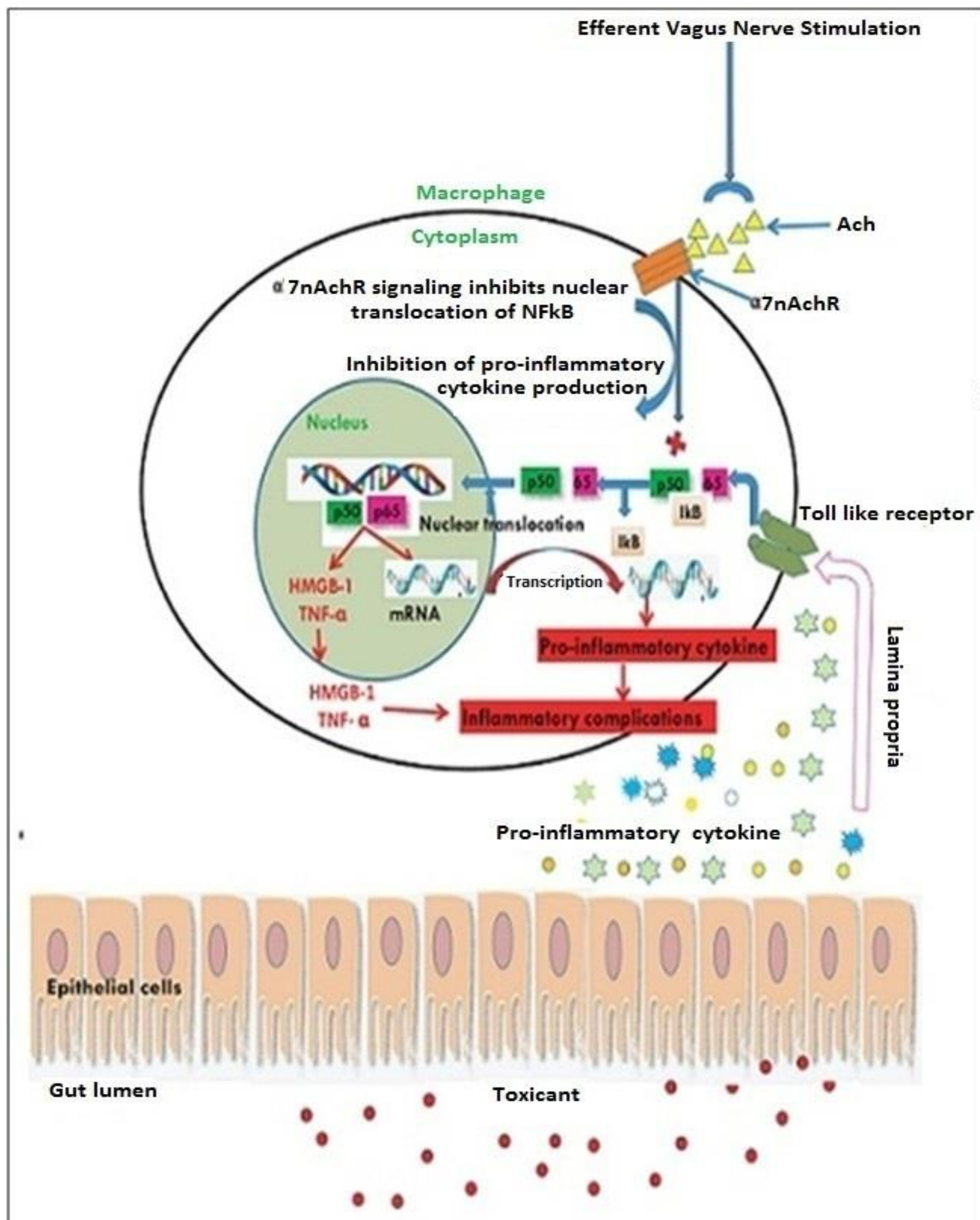
Brain controls visceral motor functions, neuroendocrine functions, pain and behavioural responses essential for survival through internal regulation system which consists of central autonomic control. The insular cortex, hypothalamus, parabrachial complex amygdale, perieqelateralgray matter, NTS and ventrolateral medulla are the essential components of the central autonomic network (CAN). CAN receive viscero-sensory as well as humoral inputs. Viscero-sensory inputs relayed to NTS while humoral inputs relayed via circum ventricular organs. The CAN responses to information received by insula cortex , the prefrontal cortices and anterior cingulated (Ee 1993). CAN have ability to modulate ANS.

Research Envisaged

ANS consider as a major player of inflammation regulation through immune system (Bellinger DL , Millar BA, Perez S, Carter J, Wood C, ThyagaRajan S, Molinaro C, Lubahn C 2008). ANS and immune system linked together through bidirectional way of vagus nerve (Willemze RA , Luyer MD , Buurman WA 2015). Nervous system and immune system work in an interdependent manner. Vagus nerve play a major role in controlling inflammation as immunity associated reflex control arc (Lv et al. 2000). Such type of interlinking results in regulation of innate immunity, which play a crucial role in modulation of inflammatory responses through humoral along with neuronal pathways (Rosas-Ballina M 2009; Matteoli and Boeckxstaens 2013; L

2004). Therefore, a hard weird connection is found between immune system and nervous system, closely interacting for regulation of inflammation through release of Ach called cholinergic anti-inflammatory pathway (CAP).

Figure 1: Schematic representation for the effect of taVNS on CAP.



Inflammatory/toxic substances invoke series of pro-inflammatory signals to bind with toll like receptors on the cellular membranes. Binding to the toll like receptors mediates the translocation of NFκB to nucleus and release of pro-inflammatory mediators. Transcutaneous auricular vagus nerve stimulation (taVNS) can felicitate the Ach release to act upon the α7 nicotinic acetylcholine receptor (α7nAchR) receptor, which in turn inhibits the translocation of NFκB to nucleus and therefore curtails the inflammatory signalling (Figure 1)

CAP is a major pathway for regulation of inflammatory cascades. Recently Tracey et al explained the anti-inflammatory role of efferent vagus nerve (KJ 2008).

Various factors are responsible for stimulation of vagus nerve including endotoxins, pro-inflammatory cytokines and lipopolysaccharide (LPS) like exotoxins. Signals generated in response to such toxins passes to the brain. In brain these signals are analysed and anti-inflammatory signals are generated, and transmitted to celiac ganglia via efferent arm of vagus nerve. After originating in DMN efferent vagus nerve connected with splenic nerve in celiac plexus and passes anti-inflammatory signals to spleen. Splenic nerve terminated in the spleen and its terminals releases nor-epinephrine. Nor-epinephrine activates specific T-lymphocytes as expressed in choline transferase cells. Choline transferase cells move to macrophages and results in secretion of Ach. Ach activate nicotinic receptors in macrophages in particular alpha seven subunit. Activation of nicotinic receptors interferes with I-κB phosphorylation and NFκB nuclear translocation followed by down regulation of pro-inflammatory cytokine expression with maintenance of anti-inflammatory cytokine levels.

As elaborated above, autonomic nervous system play a key role in regulation of inflammation through CAP. Vagus nerve is an essential component of both of pathways for their functions.

Chemotherapy and surgery are the available treatments for CRC. Chemotherapy is non- specific method, which non- specifically affects non-cancerous tissues and could results in side effects, which could be lethal. Surgical treatments are invasive and painful. Therefore, non-drug and non-surgical therapy for management of CRC is the need of the day.

Previous studies have claimed the vagus nerve stimulation in regulation of inflammation associated complications such as LPS induced inflammation and colitis (Zhao et al. 2012; Sun et al. 2013). Recently, transcutaneous auricular vagus nerve stimulation (taVNS) has been proved to be a effective strategy in the management of depression (Rong et al. 2012).

On considering the immuno-regulatory role of vagus nerve and clinical success taVNS in management of inflammatory disorders along with the advantage of being a non-invasive therapy the present study was proposed to study the effect of taVNS on CRC in animal models. To substantiate the role of α7nAchR in CRC and potential role of taVNS in the same, a selective agonistic PNU 282987 (PNU) of α7nAchR was also used in the study. All in all, we hypothesized that anti-inflammatory effects of taVNS are mediated through α7nAchRand the same could be potential therapeutic alternative for management of CRC. Since taVNS is a clinically validated non-invasive, the proposed hypothesis holds as upper edge over currently available painful and non- specific therapeutic regimes for management of CRC.

Literature review

- **Paulon et al (2017)** explored the application of non invasive vagus nerve stimulation (nVNS) for treatment of gastro paresis. In this study gastro paresis cardinal symptom index was used to grade the disease. 2 week and 3 week grading was noted for disease and treatment respectively. For

treatment, nVNS device was used. Treatment was self-administered using an nVNS device (gamma Core, electro Core) and consisted of 120 s stimulations to the vagus nerve in the neck (two stimulations to each side three times daily during weeks 1 and 2; three stimulations to each side three times daily during week 3 and beyond). Response was defined as a ≥1 point decrease from baseline in GCSI score. The results of the study indicated the beneficial effects of nVNS refractory gastro paresis (Paulon et al. 2017).

- **Saini and Sanyal (2015)** explore the role of cell cycle regulatory proteins and proinflammatory transcription factor in 1,2-dimethyl hydrazine (DMH) induced colon carcinogenesis. C-phycocyanin in combination with piroxicam was used as treatment drug. Colon cancer was induced by weekly administration of DMH at dose of 30 mg/kg for 6 weeks. Gene expression and protein expression were used to evaluate the cell cycle regulators, NFκB (p65) pathway and proliferating cell nuclear antigen while terminal deoxy nucleotidyltransferased UTP nick end labelling and apoptotic bleb assay were used for study of apoptosis. The over expression of cyclin D1, cyclin 1, CDK2 and CDK4 were noted after DMH treatment. The treatment with piroxicam and c-phycocyanin downregulation them and promoted cell cycle arrest. The piroxicam and c-phycocyanin activated p53 and mediated apoptosis. As per above discussion it was concluded that dysregulated cyclin/CDK complexes involved in various types of cancers. This study also confirmed the chemo preventive role of Cyclooxygenase-2 inhibitors (piroxicam and c-phycocyanin) against DMH induced colon carcinogenesis(Saini and Sanyal 2015).

- **Clancy et al (2014)** investigated effect of taVNS on autonomic function in healthy volunteers. They evaluated autonomic function by using heart rate variability (HRV) and microneurography. Stimulation performed by using transcutaneous electrical nerve stimulation machine and electrodes with modified surface. Volunteers received either active or sham stimulations. Study resulted in significant increment in HRV and shifting of cardiac autonomic function towards parasympathetic domain. Microneurographic recordings affirms in reduction of the frequency and incidence of muscular sympathetic nerve activity. Study conclude that taVNS can enhance HRV and diminish the sympathetic activity , which is desirable in conditions associated with increased sympathetic outflow (C. Ja et al. 2014).
- **Zhao et al (2012)** explored the protective role of taVNS for endotoxaemia in rats against LPS induced inflammation. They used vagus nerve stimulation, ta-VNS, or transcutaneous electrical acupoint stimulation on ST36 to modulate the inflammatory response. The levels of pro-inflammatory cytokine in serum and tissue NFκB (p65) was detected in endotoxaemic anesthetized rats. Study resulted in suppressive effect of VNS, taVNS over serum pro-inflammatory cytokines levels (TNF-α, IL-1β, (IL-6) along with as well as NFκB of lungs tissue. Stimulation of ST36 also resulted in decreased levels of LPS-induced high NFκB and TNF-α signalling without any significant effects on pro-inflammatory cytokine IL-1β and IL-6 levels. After vagotomy or treatment with α7nAChR antagonist, none of taVNS and ST 36 stimulation could effective in suppression of LPS-induced TNF-α and NFκB. The study confirms the role of α7nAChR-mediated CAP pathway against LPS induced inflammatory responses (Zhao et al. 2012).

- **Wang et al (2003)** reported essential regulatory role of nicotinic acetylcholine receptor α7 subunit in regulation of inflammation regulation. The wild type and α7 deficient mice were used in this study. The TNF-α production was measured in α7 deficient mice to investigate the role α7 in cholinergic anti-inflammatory pathway.

  After endotoxin administration the serum level of TNF-α in α7 deficient mice was significantly higher than wild type. Higher level of TNF-α was also reported in liver and spleen of α7 deficient animal. These findings give a clearly indicating an essential role of α7 in inflammation regulation. Knockout mice derived macrophages, produced TNF-α in response to Ach and nicotine. These findings give a clearly indicating the expression of macrophages is essential of cholinergic suppression of TNF-α production and α7 play an important role for this (H. Wang et al. 2003).

- **Tracey (2002)** explores the anti-inflammatory reflex. Inflammation is a protective and local response against toxins and infections. Since deficiency or excess of the inflammatory response, both result in lethal complications including shorting of life and death. So it must be fine regulated precisely. Previous studies established the role of cholinergic neurons in inhibition of acute inflammation. Increase understanding of anti-inflammatory reflex and CAP may result in development of physiological pathways and development of therapeutic strategies. It may be possible to activate neural anti-inflammatory mechanisms either by chemical method or by physiological method that lead in initiation of signals in proximal part of the pathway in the nervous system. VNS is one of the method which used to activate the anti-inflammatory and control of inflammatory responses by suppression of pro-

inflammatory cytokine production (Tracey 2002).

Aim and objectives

The proposed study was aimed to investigate the potential of vagus nerve stimulation in treatment of colon carcinogenesis, with following specific objectives are likely to be achieved during the course of the study:

Objective 1

To investigate the effect of taVNS on chemical induced colon carcinogenesis.

Objective 2

To enumerate how vagus nerve activity can modulate the mitochondrial apoptotic pathway in colon carcinogenesis.

Objective 3

To examine the role of α7nAchR agonist alone and in combination with taVNS on colon carcinogenesis.

Objective 4

To examine the role of α7nAchR agonist alone and in combination with taVNS on mitochondrial apoptosis.

Plan of work

1. Literature survey and procurement of materials.
2. Animal studies
    - **a.** Hemodynamic studies
        - ECG analysis
        - HRV studies
    - **b.** Physiological studies
        - Weight variation
        - pH

- Total acidity
- Aberrant crypts

c. Study of antioxidant markers

- Thiobarbituric acid reactive substances (TBARs)
- Superoxide dismutase (SOD)
- Catalase
- Protein carbonyl (PC)
- Glutathione (GSH)

d. Morphological evaluation

- Methylene blue staining
- H& E staining
- Scanning electron microscopy (SEM)
- Caspase 3 and caspase 8 evaluation

e. Immunoblotting for quantification of proteins associated with mitochondrial apoptotic pathway and cholinergic anti-inflammatory pathway.

- Bcl-2
- Bcl-xl
- BAX
- BAD
- VDAC
- Cytochrome-c
- APAF-1
- Procaspase-9
- α7nAchR

- NFκB
- TNF-α
- HMGB1

f. qRT-PCR analysis

g. ^{1}H NMR study

h. Data compilation and statistical analysis

CHAPTER 2

DRUG PROFILE

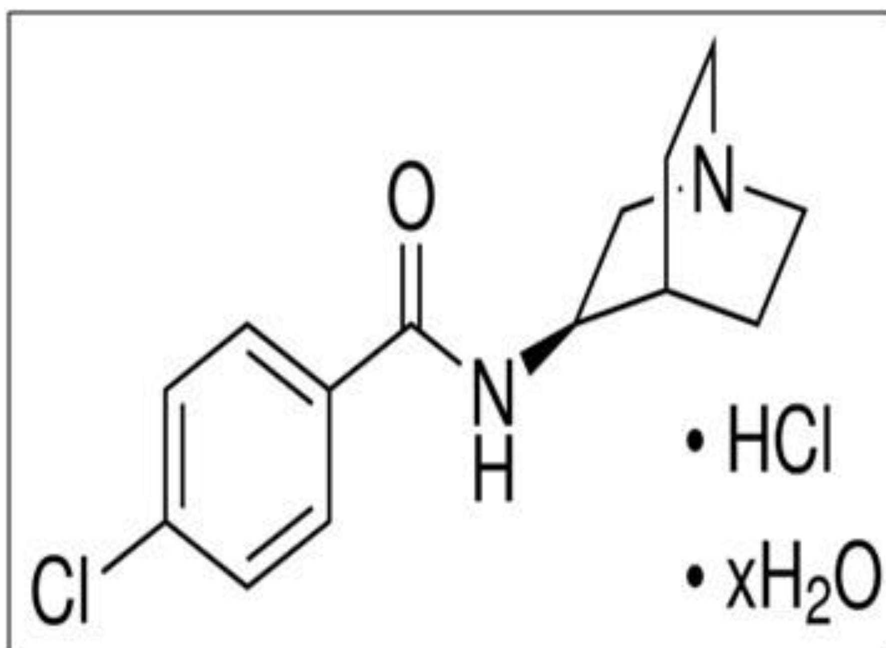
Drug profile

PNU [*N*-[(3*R*)-1-azabicyclo [2.2.2] oct-3-yl]-4-chlorobenzamide hydrochloride] is a selective α7nAchR agonist, which bind and activate α7nAchR to produce biological response This compound reported for high affinity with rat α7nAChR (K_i = 26 nM) and activity at the α7-5-HT_3 chimera (EC_{50} = 128nM). It showed negligible blocked of α1β1γδ and $\alpha_3\beta_4$ nAChRs. PNU found inactive at all tested monoamines, muscarine, glutamate and GABA receptors with exception of 5-HT_3 receptors. Nootropic action of PNU was well reported. Derivatives of PNU targeted for schizophrenia treatment. Recent studies reported protective action of PNU in hepatic ischemia-reperfusion injury through inhibition of NFκB and HMGB-1 expression in mice. PNU causes excessive inhibition of hERG antitareget, not suitable for human use. PNU has been shown to initiate signalling that lead to adult neurogenesis in mammals. Recently PNU reported for reversal of stress in animal model of Alzheimer's disease (Li F , Chen Z, Pan Q, Fu S, Lin F, Ren H, Han H, Billiar TR, Sun F 2013; Hajos 2004; VICENS and Luis HEREDIA 2017).

- Chemical name : N-*(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide*
- Formula: $C_{14}H_{17}ClN_2O.Hcl.XH_2O$
- Molecular weight: 264.75
- Purity: ≥ 98% (HPLC)
- Storage: 2-8°

Recently PNU was reported for its effectiveness in Parkinsonism associated neuroinflammation and MPTP induced nigral dopaminergic cell loss in mice. Neurodegeneration of dopaminergic neurons take place in Parkinson's disease which resulted in neuroinflammatory process in substantianigra. Cholinergic anti-

inflammatory signalling pathway n positively modulates the inflammatory processes. α7nAChR is a major component of cholinergic anti-inflammatory pathway.



PNU 282987

PNU is a selective agonist of α7nAChR and treatment with PNU resulted in attenuation of neuroinflammation in MPTP lesioned substantia nigra. (Stuckenholz et al. 2013)

CHAPTER 3

MATERIALS

&

METHODS

Materials and methods

Materials

Drugs and chemicals

DMH (LOT:A0251168) was purchased from Acros organics, New Jersey, USA.; PNU (053M4723V) was purchased from Sigma Aldrich Co. St. Louise Mo 63103 USA.; hematoxylin (Himedia,S058); eosin (Himedia,S007); RIPA lysis buffer (Amresco, N653); protein assay kit (Amresco,M173); bovine serum albumin (BSA) (Genetix,PG-2330); transfer buffer (Genetix, GX-9411AR), trizol reagent (Sigma-T9424), cDNA synthesis kit (Genetix-K1612). Caspase 3 (SC-4263) and caspase 8 (SC-4267) assay kits were procured from Santacruz Biotechnology Inc., California, Delaware. Primary antibodies for Bcl-xl (MA-5-15142), Bcl-2 (SC-7382), BAX (SC-23959), BAD (SC-8044), VDAC (SC-390996), cytochrome c (SC-13561), Apaf-1 (SC-65891), procaspase-9 (SC 73548), HMGB-1 (SC-56698), α7nAchR (SC-5544), NFκBp65 (MA5-1616) and TNF-α (SC-1350) All others chemicals were of molecular biology grade and purchased from Genetix Biotech Asia Pvt. Ltd, New Delhi.

Equipment's

Table 1: Lists of equipment's used

S. No.	Equipment	Manufacturer and Model
1.	Cooling centrifuge	Eppendorf India Limited, Chennai 5418R
2.	Vortex shaker	Remi Mumbai CM101
3.	Refrigerator	Godrej, Lucknow
4.	Homogenizer	Remi, Mumbai RQT-127A
5.	Micro volume Spectrophotometer	Agilent Technologies, Mumbai Carry 500
6.	Weighing balance	Sartorius, Mumbai BSA224S-CW
7.	pH meter	LMPH-10, Labman Scientific Instruments, Lucknow
8.	Micropipette	Genetix Biotech Asia Pvt. Ltd. New Delhi
9.	Deep freezer	Celfrost, BFS150,Lucknow
10.	Micro plate Reader	Bio-Rad Laboratories Inc. Model 680XR
11.	Bio-amplifier (ML-136)	AD Instruments Pty Ltd Bella Vista New South Wales, Australia
12.	Channel power lab (ML-826)	AD Instruments Pty Ltd Bella Vista New South Wales, Australia
13.	Digital biological microscope	N-120, BR-Biochem Life Sciences, New Delhi
14.	Scanning electron microscope	JEOL JSM-6490LV
15.	SDS-PAGE	GX-SCZ2, Genetix Biotech Asia Pvt. Ltd. New Delhi
16.	Semidry transfer unit	GX-ZY3, Genetix Biotech Asia Pvt. Ltd. New Delhi

Animals

The male *albino wistar* rats (120–140 g) were obtained from the central animal house facility and maintained under standard laboratory conditions, temperature (25±1°C) with 12 h light /dark cycle. Animals were fed with standard animal diet and water ad *libitum.*

The study was approved by institutional animal ethics committee (IAEC) of Sam Higginbottom University of Agriculture, Technology and Science - A Deemed University (approval no. IAEC/SHIATS/PA16III/SJPG17)

Experimental protocol

(a)Transcutaneous vagus nerve stimulation regulates cholinergic anti-inflammatory pathway to counteract 1, 2-dimethyl hydrazine induced colon carcinogenesis in *albino wistar* rats

Animals were randomly chosen and grouped in ten groups of eight animals each. Carcinogenicity was induced by weekly injection of DMH (30 mg/kg, s.c) and taVNS was applied at various intensities through auricular chonchal region.

For taVNS, animals were stimulated with specific stimulating parameters including pulse width (ms), frequency (Hz), voltage (v) and duration (min). Stimulation started from 4th day of DMH administration with exception of taVNS control and dummy VNS groups.

Group I (Control): Animals of this group administered with 1 mM EDTA–saline (2ml/kg/day, s.c) for 6 weeks.

Group II (taVNS control): Animals received taVNS (1.0 ms, 6Hz, 6v and 40 min. / week), for 6 weeks without any other treatment.

Group III (DMH control): DMH (30 mg/kg/week, s.c.) was administered for 6 weeks to induce the CRC. DMH was dissolved in 1 mM EDTA-saline and maintained its pH 7.0 by using NaOH prior to administration [38,39]

Group IV (DMH + taVNS1) : Animals received taVNS (1.2 ms, 4Hz,6v,33.33 min. / week) in conjugation to DMH administration (30 mg/kg/week, s.c.) for 6 weeks.

Group V (DMH + taVNS2): Animals received taVNS (1.0 ms, 2 Hz, 3 v, 40 min. /week) along with DMH (30 mg/kg/ week, s.c.) treatment for 6 weeks.

Group VI (DMH + taVNS3): Animals received taVNS (1.0 ms, 6 Hz, 6v, 40 min. /week) in conjugation with DMH (30 mg/kg/ week, s.c.) administration for 6 weeks.

Group VII (DMH + taVNS4): Animals received taVNS (1.4 ms, 6Hz, 2v, 26.66 min/week) along with weekly administration of DMH (30 mg/kg/week, s.c.) for 6 weeks.

Group VIII (DMH + taVNS 5): Animals treated with taVNS (1.6 ms, 1 Hz, 5v, 20 min./week) in conjugation with DMH (30 mg/kg / week, s.c.) for 6 weeks.

Group IX (Standard chemotherapy) : Animals treated with Leucovorin followed by 5-Flourouracil (25 mg/kg, i.p. at day 1st , 3rd , 5th , 7th and 10th after 6 week administration of DMH (30 mg/kg / week, s.c.).

Group X (Dummy VNS): Only stimulating electrodes placing on the chonchal region of ear pinna without any other treatment.

Weight variations between groups noted on weekly basis for 6 weeks.

All methods were performed as per the guidelines of CPCSEA; Department of animal welfare; Government of India.

Table 2: Treatment schedule

<table>
<tr><th>S. No.</th><th>Group</th><th colspan="4">Treatment</th></tr>
<tr><td>1.</td><td>Control</td><td colspan="4">1 mM EDTA- saline, 2 ml/kg, s.c.</td></tr>
<tr><td>2.</td><td>taVNS control</td><td colspan="4">Pulse width -1.0 ms , Frequency-6.0 Hz, Voltage – 6v, Duration – 240 min.</td></tr>
<tr><td>3.</td><td>DMH control</td><td colspan="4">DMH (30 mg/kg, s.c.)</td></tr>
<tr><th colspan="2">Transcutaneous auricular vagus nerve stimulation (taVNS)</th><th>Pulse width (ms)</th><th>Frequency (Hz)</th><th>Voltage (V)</th><th>Duration (Min.)</th></tr>
<tr><td>4.</td><td>DMH + taVNS 1</td><td>1.2</td><td>4.0</td><td>6.0</td><td>33.33 min/week (200 min. for 6 weeks)</td></tr>
<tr><td>5.</td><td>DMH + taVNS 2</td><td>1.0</td><td>2.0</td><td>3.0</td><td>40.00 min/week (240 min. for 6 weeks)</td></tr>
<tr><td>6.</td><td>DMH + taVNS 3</td><td>1.0</td><td>6.0</td><td>6.0</td><td>40.00 min/week (240 min. for 6 weeks)</td></tr>
<tr><td>7.</td><td>DMH + taVNS 4</td><td>1.4</td><td>6.0</td><td>2.0</td><td>26.66 min/week (160 min. for 6 weeks)</td></tr>
<tr><td>8.</td><td>DMH + taVNS 5</td><td>1.6</td><td>1.0</td><td>5.0</td><td>20.00min/week (120 min. for 6 weeks)</td></tr>
<tr><td>9.</td><td>Standard chemotherapy</td><td colspan="4">Leucovorin followed by 5-Flourouracil (25 mg/kg, i.p.) + DMH (30 mg/kg, s.c.)</td></tr>
<tr><td>10.</td><td>Dummy control</td><td colspan="4">Only stimulator without any current</td></tr>
</table>

(b)Effect of α7nAchR agonist alone and in combination with transcutaneous vagus nerve stimulation on mitochondrial apoptosis against 1, 2-dimethyl hydrazine induced colon carcinogenesis in *albino wistar* rats.

Experimental animals were randomly allocated to seven groups (n=6) designated as I-VII. Carcinogenicity was induced by weekly injection of DMH (30 mg/kg, s.c) and taVNS was applied at various intensities through auricular chonchal region. For taVNS, animals were stimulated with specific stimulating parameters including pulse width (ms), frequency (Hz), voltage (v) and duration (min). Stimulation started from 4th day of DMH administration with exception of taVNS control and dummy VNS groups..

Group I (Control): Animals of this group administered with 1 mM EDTA–saline (2ml/kg/day, s.c) for 6 weeks.

Group II (taVNS control): Animals received taVNS (1.0 ms, 6Hz, 6v and 40 min./ week), for 6 weeks without any other treatment .

Group III (DMH control): DMH (30 mg/kg/week, s.c.) was administered for 6 weeks to induce the CRC. DMH was dissolved in 1 mM EDTA-saline and maintained its pH 7.0 by using NaOH prior to administration (Tanwar, Vaish, and Sanyal 2009; Sanyal et al. 2015).

Group IV (Standard chemotherapy) : Animals treated with Leucovorin followed by 5-Flourouracil (25 mg/kg, i.p. at day 1st , 3rd , 5th , 7th and 10th after 6 week administration of DMH (30 mg/kg / week, s.c.).

Group V (Dummy VNS): Only stimulating electrodes placing on the chonchal region of ear pinna without any other treatment.

Group VI (DMH+PNU) : DMH(30 mg/kg/weeks,.c.)+ PNU (0.5 mg /kg/week, i.v. for 3 weeks),

Group VII (DMH+PNU+taVNS): DMH (30mg/kg/week, s.c.)+ PNU (0.5 mg /kg/week, i.v. for 3 weeks) + taVNS (pulse width - 1 ms, frequency- 6 Hz, voltage – 6 v, duration – 40 min, weekly)

taVNS

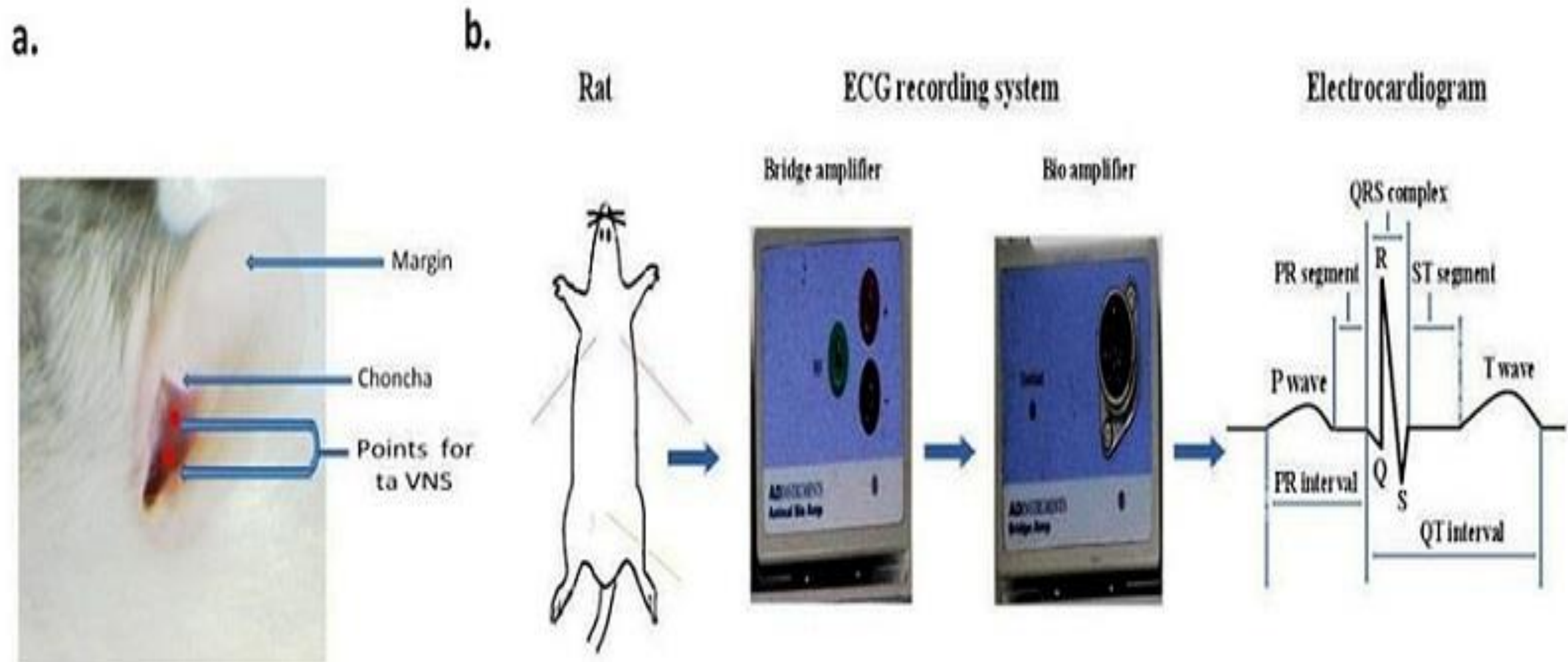
Animal nerve stimulating electrode (MLA0320, AD Instruments, Australia), consisting of two exposed gold plated brass electrodes with rounded ends, was used for taVNS. Each electrode was having 2mm diameter and separated with each other by 3mm. The animals were anaesthetized using ketamine hydrochloride (100 mg/kg, i.m.) and diazepam (5 mg/kg, i.m.) combination followed by mounting on a wax tray and electrodes were placed on the auricular chonchal region for stimulation (Figure 1a) (Rong et al. 2012; Li et al. 2014). The electrode leads were connected with power lab system 2/26 (AD Instruments, Australia) for stimulations. ECG and HRV were recorded (Power lab system 2/26, AD Instruments Australia) during stimulation for supervision of hemodynamic variations. To improve electronic conduction, saline was applied on skin surface. The stimulation started from 4th day after DMH treatment and continued weekly up to 6 weeks along with DMH. The stimulation parameters are detailed in table 2(Li et al. 2014; Kong et al. 2012).

On 42nd day, animals were recorded for ECG and HRV. On 43rd day blood was collected from retro-orbital plexus and incubated (37°C, 1h) followed by centrifugation to collect serum. Afterwards the animals were sacrificed under light ether anaesthesia. The colon tissue of animals was collected by securing the both ends with a surgical suture to prevent drainage of the colonic content.

Hemodynamic changes

The positive and negative platinum hook electrodes were placed on the skin of left and right side of thorax and neutral electrode on skin of peritoneal region of anesthetized rat. The other ends of electrodes were connected with power channel (ML-826) and Bio-amplifier (ML-136) for conversion of analogue signals to digital. The record ECG signals were analysed offline, using Lab Chart Pro-8. Analysis of hemodynamic raw data started with ensuring the correctness of R wave followed by calculation of heart rate (HR) and other ECG parameters. Similarly, the time and frequency domain parameters for HRV were calculated from the ECG signals using Lab Chart Pro-8, following the method elaborated elsewhere (Shintaku et al. 2014; Xiong et al. 2016) (Figure 2).

Figure 2: Auricular transcutaneous auricular vagus nerve stimulation & Hemodynamic changes.



a: Locations of stimulation points on rat's auricular surface for taVNS. The auricular choncha is the region of ear, where major transcutaneous distribution of vagus nerve occurs. The stimulation points for taVNS are located in this region.
b: Placement of electrodes and recording of ECG in experimental animals.

Weight variation

Weight variation within treatment groups was observed and calculated using the formula I (Mishra et al. 2016).

Weight variation (%) $= \frac{\text{Finalweight} - \text{Initialweight}}{\text{Final weight}} \times 100$ (I)

Estimation of pH and total acidity

The colonic content was collected and evaluated for pH estimation using pen type pH meter (Hanna Instruments, HI98107). Total acidity was calculated using a previously described procedure (Giri et al. 2015; A. Kumar et al. 2016).

Aberrant crypt foci (ACF)

Longitudinally opened colonic tissue was washed with normal saline and fixed by placing it between two layers of whatman paper for 24 h in 10% formalin. Subsequently, tissue was stained with 2% methylene blue and visualised through light microscope. The ACF was visualised and counted in 5 independent frames using the method elaborated previously by our laboratory (Bird 1987; Mishra et al. 2016).

Morphological evaluation

SEM (JEOL-JSM-6490LV) was used to study the morphological changes on the colonic mucosa. Samples were prepared by fixation of tissues in 2.5% glutaraldehyde for 6 h at 4°C. Tissues were washed with 0.1 M phosphate buffer; post fixed and dehydrated using the method described elsewhere. The tissues were mounted on aluminium stub with adhesive tape and visualise at magnification of 500 X and 2000 X (T. et al. 1984; Sammi et al. 2018).

Histopathology

H&E staining was used to study the histopathology of colonic tissues. The tissues were fixed overnight in paraformaldehyde with subsequent dehydration using

isopropanol and xylene. Dehydrated tissues were fixed in paraffin wax and sectioned (0.5 mm) using microtome. Sections were stained with H&E, and visualized under digital biological microscope at magnification of 40X (N120,BR-Biochem Life Sciences, New Delhi, India) (Serafino et al. 2014).

Antioxidant markers

The colon tissues (10% w/v) were homogenized in 0.15% KCl and centrifuged at 10,000 rpm. The supernatant was used for biochemical estimations for TBAR's, SOD, catalase, GSH and PC using previously established methods at our laboratory (Gupta et al. 2014; S. Kumar et al. 2014; Raj et al. 2014; Gautam et al. 2016) .

Western blotting

The colon tissue was lysed in RIPA lysis buffer to obtain total protein lysate and quantified using Bradford reagent. The proteins were resolved through 12.5% SDS-PAGE and transferred to PVDF membrane (IPVH 00010 Millipore, Bedford, MA US) using a method established at our laboratory (Roy et al. 2017).

Following the transfer, membrane was blocked with solution of BSA and skimmed milk in TBST (3%) for 3 h. The blocked membrane was incubated overnight with primary antibodies for Bcl-xl (MA-5-15142), Bcl-2 (SC-7382), BAX (SC-23959), BAD (SC-8044), VDAC (SC-390996), cytochrome-c (SC-13561), Apaf-1 (SC-65891), procaspase-9 (SC 73548), HMGB-1 (SC-56698), α7nAchR (SC-5544), NFκBp65 (MA5-1616) and TNF-α (SC-1350) at 4°C. After incubation with primary antibody, membrane was washed thrice with TBST and once with TBS. Subsequently, membrane was incubated with the HRP conjugated secondary antibody including anti-rabbit (SC-2030), anti-goat (SC-2020), anti-mouse (31430, Pierce Thermo Scientific, USA) (1:5000 dilutions) for 3 h.

The membranes was developed by using enhanced chemiluminescence substrate (Western Bright ECL HRP substrate, Advansta, Melanopark, California, US) in gel dock system after three and single washing with TBST and TBS respectively. The protein bands were quantified by densiometric digital analysis using Image J software. β-actin (MA5-15739-HRP) was used as a standard reference (Roy et al. 2017).

qRT-PCR

Primer quest, tool from the IDT DNA technologies website (http://www.idtdna.com) was used for online primer designing for real time. Size of amplicon was kept between 100 and 200 base pairs. GC% was kept above 50% and melting temperature was kept between 58°C and 62°C. The sequences of forward and reverse primers used for qRT-PCR are detailed in table 3 (Liu, Mizuta, and Matsukura 2004).

Total RNA was extorted from colon tissues by the use of trizol reagent as according to manufacturer instructions. Tissues were meshed in 250 µl trizol reagent by using micro pestle and volume was made up to 1 ml followed by addition of 200 µl chloroform and vortexing for 5 min.

Tissue suspension was centrifuged at 14,000 rpm, 4°C for 15 min. The aqueous phase was separated and transferred to a fresh vial, with subsequent centrifugation at 14000 rpm for 10 min. The pellets were washed twice with chilled ethanol and dissolved in 15µl of 1% DEPC water. The samples were quantified through nanodrop (Qua Well Q5000).

1µg of tissue RNA was used for cDNA synthesis, which was used as a template for qRT -PCR reaction using the method described previously by us. β actin was used as a house keeping control (Roy et al. 2017).

Assay for caspase 3 and caspase 8

Fluorometric assays for caspase 3 and 8 were performed in amber coloured 96 well plates using the protocol provided by the manufacturer. The equal volume of serum taken from all treatment groups was diluted with reaction buffer followed by addition of dithiothriol (DTT) to a final concentration of 10 mM. 5μl of IETD-AFC/DEVD-AFC substrate was added to the reaction mixture and incubated for 1 h at 37°C. The formed free AFC levels were measured in a plate reader at 400 nm excitation and 505 emissions. The results were expressed as fluorescence units/mg of protein (Brown et al. 2015; Kim et al. 2003).

1H-NMR based serum metabolomics

Sample preparation for NMR spectroscopy

Sample preparation and NMR data acquisition on all samples were executed using the methodology described previously (Roy et al. 2017). Briefly, serum sample was mixed with an equal volume of 0.9% saline sodium-phosphate buffer (20 mM, pH 7.4, prepared in D_2O), the sample mix was centrifuged at 10,000 rpm for 5 min, 450 μL of the clear supernatant was aliquot in a 5 mm NMR tube with a co-axial insert containing the known concentration 0.1 mM of 3-trimethylsilyl-(2,2,3,3-d4)-propionic acid (TSP) for NMR data acquisition. It (TSP) is an external standard to assist metabolite quantification and also to provide a deuterium field/frequency lock for NMR experiment. Following this,1D^{1}HNMR spectra were acquired using the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence (cpmgpr1d, standard Bruker pulse program)at 300 Kelvin (K)on a Bruker Biospin Avance-III 800 MHz NMR spectrometer equipped witha Cryo Probe. Additionally, 2D Jres NMR spectra were

also recorded to aid the assignment of metabolites using pool samples from each group.

Data Processing and Multivariate Analysis

All the obtained spectra were processed using Topspin-3.5 (Bruker GmbH, Rheinstetten, Germany) followed by manual phase and baseline-correction. Next, all the spectra were referenced to lactate chemical shift (δ=1.3102) to provide spectral homogeneity and compensate for minor shifts in the spectra, throughout the samples. All the processed spectrawere reviewed for acceptabilityby visual inspection of an overlay of all the spectra, before importing into the AMIX software (Bruker) for binning/bucketing. Each spectrum in the region 0.7–8.5 ppm was segmented into 0.02-ppm chemical shifts bins (buckets), the region distorted due to water suppression (4.65–5.01 ppm) was omitted. The resulting binned CPMG datasetwas used for multivariate analysis in Metabo Analyst an open access web-based tool for metabolomics data analysis, visualization, and interpretation(Xia et al. 2009, 2015). Standard procedures of multivariate and univariate statistical analysis were used to maximize the extraction of relevant information from the CPMG datasets. For multivariate analysis both combined and pairwise- principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and orthogonal projection to latent structure with discriminant analysis (OPLS-DA) was performed to identify and visualize class separationbetween the control and the treated groups. The PLS-DA model was used for pairwise analysis to identify the discriminatory metabolites based on their VIP values (variable importance on projection scores) (i.e., VIP≥1 in general). The goodness-of-fit parameters R^2 and Q^2, which relate to the explained and predicted variance, respectively, were used to evaluate the PLS-DA model performance. Also, statistical reliability of the obtained OPLS-DA models was

rigorously validated using permutations testing (n = 100). Further, univariate analysis was used to assess the significance of the change in the metabolic profile for pairwise and combine analysis (t-test and ANOVA respectively), p-value < 0.05 isconsideredas the measureof statistical significance.

Statistical analysis

Results are presented as mean ± SD and analysed by one-way ANOVA followed by Bonferroni test for the possible significance identification between the various groups .[a]p<0.05, [b]p<0.01, [c]p<0.001 were considered statistically significant. Statistical analysis was performed using Graph Pad Prism software (5.02).

ECG and HRV, reanalysed by using Metabo Analyst. Metabo Analyst is a web based data analysis tool. In this results obtained in graphical format, easy to understand. Metabo Analyst can use for various analysis including biomarker analysis, pathway analysis but here used statistical analysis. Raw data was saved in comma separated values format and uploaded as spectral bins followed by data integrity check, data filtering and normalization of data (pareto scaling). Finally multivariate analysis of data was performed by using PCA and PLS-DA method (Xia et al. 2009).

Table 3: Sequence of forward and reverse primers used for quantitative RT-PCR

S.No.	Primer	Sequence
1.	Bcl-2 F	GTGGATGACTGAGTACCTGAAC
2.	Bcl-2 R	GAGACAGCCAGGAGAAATCAA
3.	Bcl-xl F	CCCTCGTATCTGGAAGCCAC
4.	Bcl-xl R	CAGCGGAGACCTCGTTTTCT
5.	BAX F	TGCTACAGGGTTTCATCCAG
6.	BAX R	RGACACTCGCTCAGCTTCTT
7.	BAD F	CTCCGAAGAATGAGCGATGAA
8.	BAD R	ATCCCACCAGGACTGGATAA
9.	VDAC F	GGAGTTTGGTGGCTCCATTTA
10.	VDAC R	GACCTGATACTTGGCTGCTATTC
11.	Cytochrome - C F	TCCATTTCCCTTCCTTGGGC
12.	Cytochrome -C R	ATCGGGGCTGTCCAACAAAA
13.	Apaf-1 F	GAACATAGACTCCCGGGTAAAG
14	Apaf-1 R	CTTGTCTCCCAGACCCTTATTG
15.	Procaspase-9 F	GGCTCTCTGGCTTCATTCTT
16.	Procaspase-9 R	GGGTCCAGCTTCACTACTTTC
17.	α7nAchR F	AACTGGTGTGCATGGTTTCTGCGC
18.	α7nAchR R	AGATCTTGGCCAGGTCGGGGTCCC
19.	NFκB F	GGGCTACGAAGTCAAACCCA
20.	NFκB R	RTTCTCCTCAATCCGGTGACG
21.	TNF-α F	GCAGGTCTACTTTGGAGTCATT
22.	TNF-α R	GGCTCTGAGGAGTAGACGATAA
23.	HMGB-1 F	AAGACGACGAGGAGGATGAA
24.	HMGB-1 R	ACTGCGCTAGAACCAACTTATT
25.	β-actin F	TGCAGGATCGTGAGGAACAC
26.	β-actin F	AGCGTGATTGTAACGCCTGA

CHAPTER 4

RESULTS

&

DISCUSSION

Results

(a) Transcutaneous vagus nerve stimulation regulates cholinergic anti-inflammatory pathway to counteract 1, 2-dimethyl hydrazine induced colon carcinogenesis in *albino wistar* rats

Hemodynamic study

The DMH treatment was very well manifested for increase in RR interval (0.20±0.01 s), HR (380.1±29.96bpm), QRS interval (0.02±0.001 s), P (0.04±0.003 mv) and Q (0.20±0.01 mv) wave amplitude along with decrease in QT interval (0.07±0.007 s), JT interval (0.05±0.003 s), QTc complex (0.15±0.01 s) and R wave amplitude (1.35±0.12 mv) (Figure 1c, table 4). The PCA and PLS-DA analysis of the ECG signals revealed that taVNS 3, 4 and 5 could favourably regulate the ECG signals towards control. The HRV analysis revealed significant curtailment of average RR (157.80±9.15ms), median RR (158.8±10.26ms), low frequency (LF) (1.2±0.10 μs^2), high frequency (HF) (9.10±0.81 μs^2) and LF/HF (0.13±0.01) in DMH treated animals (table 5). taVNS 2 and 3 could favourably curtail the deleterious effects of DMH (Figure 3). When considering standard, treatment with taVNS more favourably restored hemodynamic changes towards normal control.

Table 4: Effect of taVNS on ECG against DMH induced colon carcinogenesis

ECG Parameters	Control	taVNS control	DMH control	taVNS 1	taVNS 2	taVNS 3	taVNS 4	taVNS 5	Standard chemotherapy	Dummy control
RR Interval (s)	0.18±0.01^{c}	0.17±0.01^{c}	0.16±0.01	0.15±0.01^{c}	0.17±0.01^{c}	0.20 ±0.02^{c}	0.17 ±0.01^{c}	0.16±0.01^{c}	0.16±0.01^{c}	0.15±0.01^{c}
HR (BPM)	330.84±24.81^{a}	355.11±9.69	380.1±29.96	386.42±23.73	349.28±23	299.04±25.43^{c}	357.16±28.5	374.01±29.92	373.3±17.13	411.46±25.66
PR Interval (s)	0.04±0.002	0.04±0.003	0.04±0.003	0.03±0.002^{c}	0.04±0.001	0.04±0.002	0.04±0.003	0.04±0.003	0.04±0.001	0.03±0.002^{c}
P Duration (s)	0.01±0.001	0.01±0.001	0.01±0.008	0.01±0.0001	0.01±0.001	0.01±0.001	0.01±0.001	0.01±0.001	0.01±0.006	0.01±0.007
QRS Interval(s)	0.01±0.008^{c}	0.01±0.001^{c}	0.02±0.001	0.02±0.001	0.02±0.001	0.02±0.001^{c}	0.01±0.006	0.02±0.003	0.01±0.007^{c}	0.01±0.0
QT Interval(s)	0.09±0.01^{c}	0.05±0.003^{c}	0.07±0.007	0.06±0.004	0.14±0.01^{c}	0.16±0.01^{c}	0.06±0.004	0.05±0.002^{c}	0.05±0.003^{c}	0.05±0.002^{c}
J T Interval (s)	0.07±0.005^{c}	0.03±0.002^{c}	0.05±0.003	0.04±0.002^{c}	0.03±0.002^{c}	0.03±0.001^{c}	0.04±0.002^{c}	0.04±0.002^{c}	0.03±0.002^{c}	0.03±0.002^{c}
Tpeak T end Interval (s)	0.04±0.003^{c}	0.02±0.001^{c}	0.03±0.002	0.03±0.001	0.02±0.001	0.03±0.002^{c}	0.03±0.002^{c}	0.02±0.001^{c}	0.01±0.001^{c}	0.02±0.001^{c}
QTc (s)	0.19±0.01^{c}	0.12±0.01^{c}	0.15±0.01	0.16±0.01	0.14±0.01	0.16±0.01	0.16±0.01	0.12±0.01^{c}	0.13±0.01	0.14±0.01
P Amplitude (mV)	0.1±0.01^{c}	0.06±0.004^{c}	0.04±0.003	0.08±0.006^{c}	0.05±0.003^{c}	0.09±0.01^{a}	0.04±0.003	0.06±0.004^{c}	0.04±0.003	0.05±0.003^{a}
Q Amplitude (mV)	0.04±0.002^{c}	0.02±0.001^{c}	0.20±0.01	0.03±0.002^{c}	0.07±0.005^{c}	0.02±0.001^{c}	0.06±0.004^{c}	0.03±0.001^{c}	0.01±0.001^{c}	0.07±0.005^{c}
R Amplitude (mV)	1.80±0.15	1.17±0.09	1.35±0.12	1.17±0.09	0.24±0.02^{c}	1.77±0.15	0.97±0.07	1.17±0.09	0.76±0.05	0.94±0.07
S Amplitude (mV)	-0.14±0.01^{a}	-0.07±0.005^{c}	-0.16±0.01	-0.05±0.003^{c}	0.009±0.001^{c}	-0.14±0.01^{a}	-0.17±0.01	-0.07±0.001^{c}	-0.13±0.01^{c}	-0.12±0.01^{c}
T Amplitude (mV)	0.40±0.02^{c}	0.16±0.01^{a}	0.12±0.01	0.30±0.02^{c}	0.24±0.02^{c}	0.38±0.02^{c}	0.29±0.02^{c}	0.16±0.01^{a}	0.03±0.0001^{c}	0.02±0.001^{c}
ST Height (mV)	0.10±0.07^{c}	0.01±0.001^{c}	0.03±0.002	0.05±0.001^{c}	0.09±0.001^{c}	0.09±0.001^{c}	0.04±0.003^{c}	0.01±0.001^{c}	0.05±0.003^{c}	0.10±0.01 ^{c}

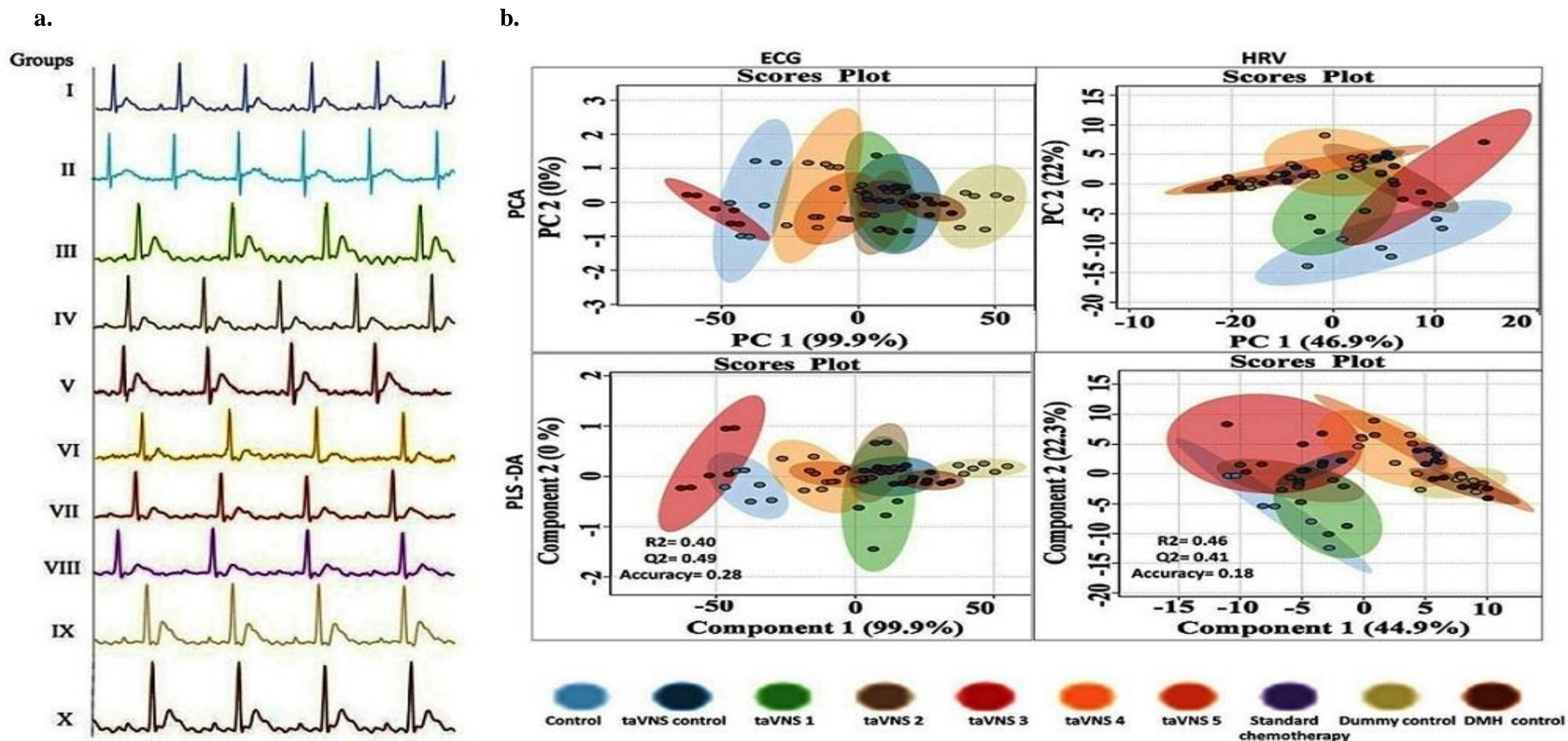
(Values are presented as Mean ±SD), each group contains 8 animals. Comparisons were made on the basis of the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the DMH control group ($^{a}p< 0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$).

Table 5: Effect of taVNS on HRV against DMH induced colon carcinogenesis

HRV	Control	taVNS control	DMH Control	taVNS 1	taVNS 2	taVNS3	taVNS4	taVNS 5	Standard chemotherapy	Dummy control
Time domain										
Average RR(ms)	181.3±14.78[a]	168.90±12.16	157.80±9.15	155.27±9.64	171.8±4.40	200.64±13.15[c]	167.99±12.97	160.42±12.63	160.72±11.72	145.82±9.21
Median RR (ms)	182±13.23[a]	170.6±13.27	158.8±10.2	156.5±11.63	172±15.29	202.4±12.37[c]	169.16±12.40	161.00±14.28	162.8±13.11	147.2±12.20
SDRR (ms)	6.22±0.50[c]	9.18±0.85[c]	3.97±0.34	2.31±0.19[c]	8.60±0.75[c]	6.57± 0.45[c]	8.58±0.96[c]	7.20±0.62[c]	7.20±0.65[c]	3.36±0.27
SDARR	3.40±0.29[c]	9.44±0.83[c]	8.50±0.95	6.07±0.17[c]	21.74±1.93[c]	8.16±0.73[c]	21.60±1.97[c]	21.604±1.82[c]	17.83±1.29[c]	1.93±0.16
CVRR(ms)	0.04±0.002[c]	0.05±0.003[c]	0.02±0.001	0.01±0.001	0.04±0.003[c]	0.03±0.002[c]	0.09±0.01[c]	0.03±0.001[c]	0.03±0.002[c]	0.02±0.001
Frequency domain										
LF(μs^2)	15.38±1.33[c]	0.14±0.01	1.2±0.10	0.27±0.02[c]	0.38±0.02	1.33±0.11[a]	9.17±0.79[c]	1.45±0.13[b]	0.64±0.05	0.82±0.06
HF (μs^2)	60.84±5.95[c]	0.84±0.07[c]	9.10±0.81	1.33±0.12[c]	1.71±0.11[c]	5.25±0.35	18.94±1.56[c]	4.97±0.37[a]	6.23±0.51	7.48±0.65
LF/HF	0.25±0.02[c]	0.16±0.01	0.13±0.01	0.36±0.03[c]	0.21±0.01[c]	0.25±0.01[c]	0.48±0.03[c]	0.29±0.02[c]	0.10±0.001	0.10±0.01
VLF(μs^2)	1.69±0.13[c]	2.77±0.25[c]	1.36±0.03	6.66±0.48[c]	3.12±0.27[c]	1.36±0.02	5.53±0.49[c]	1.38± 0.12[c]	1.70±0.13[c]	2.04±0.17[c]

Values are presented as Mean ± SD), each group contains 8 animals. Comparisons were made on the basis of the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the DMH control group ([a]p< 0.05, [b]p< 0.01, [c]p< 0.001).

Figure 3: Representative ECG recordings and analysis in response to taVNS treatment against DMH induced colon carcinogenesis



a. Representative ECG recordings of control, DMH and taVNS treatments.

b. The figure represents for 2D score plots derived from PCA and PLS-DA based analysis for ECG and HRV signals in different groups

pH, total acidity, percentage weight variation and ACF count

When scrutinized on the account of physiological parameters, DMH treatment perceived significant decrease in weight (-14.17±1.32%), non significant decrease in pH (6.31±0.56); with increase in total acidity (127.96±8.6 $mEql^{-1}$) and ACF (56±2.29 NoS). taVNS regulated the weight variation and pH significantly. The total acidity and ACF count was favourably regulated more profoundly by taVNS3. When comparing with standard, taVNS resulted in better regulation of weight variation (all taVNS groups), pH (taVNS 1 and 2) and total acidity (taVNS 1) towards control. The taVNS treatment not favourably regulated the ACF as compared with standard chemotherapy against DMH induced colon carcinogenesis (Table 6).

Antioxidant assay

When DMH treatment accounted for the oxidative stress marker upsurged TBAR' s (187.82±6.78 nM of MDA /μg of protein) and PC (106.68±10.82nM/ml unit) in comparison to control. The SOD (0.004±0.001 unit of SOD /mg of protein) and catalase (1.67±0.086nM of H_2O_2 /min. /mg of protein) levels were down-regulated with significant increase in GSH (0.68±0.07 mg %) in DMH treated animals. taVNS 2 and 3 embarked a more favourable regulation for TBARs and PC respectively. taVNS 2 and 3 regulated the GSH, SOD and catalase as well (Table 7). When comparing effects of taVNS and standard chemotherapy against DMH induced colon carcinogenesis, taVNS was more effective in regulation of TBAR's (all taVNS treatments), catalase (all taVNS treatments) and GSH (taVNS 4).

Treatment with taVNS resulted in positive regulation of PC and GSH against DMH control but comparatively less effective as standard chemotherapy.

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Table 6: Effect of taVNS on physiological parameters against DMH induced colon carcinogenesis

	Weight variation (%)	pH	Total acidity($mEql^{-1}$)	Aberrant crypts (NoS)
Control	21.22±1.75^{c}	6.97±0.62	95.97±4.5^{c}	11.6±0.87^{c}
taVNS control	22.4±1.43^{c}	6.98±0.59	95.06±5.6^{c}	16.4±1.13^{c}
DMH control	-14.17±1.32	6.31±0.56	127.96±8.6	56±2.29
taVNS 1	18.65±1.56^{C}	7.01±0.61	93.97±7.56^{c}	21.4±1.72^{c}
taVNS 2	20.95±1.62^{c}	7.00±0.63	94.74±6.7^{c}	22±0.96^{c}
taVNS 3	24.42±1.95^{c}	6.98±0.56	95.45±8.12^{c}	28±1.64^{c}
taVNS 4	26.8±2.18^{c}	6.91±0.61	100.97±7.3^{c}	30.2±1.12^{c}
taVNS 5	30±2.31^{c}	6.85±0.62	103.36±6.95^{c}	38±2.70^{c}
Standard chemotherapy	12.4±0.98^{c}.	6.99±0.57	94.37±5.72^{c}	13.8±1.12^{c}
Dummy control	24.84±2.12^{c}	6.92±0.54	100.73±7.23^{c}	17.4±0.79^{c}

(Values are presented as Mean ± SD), each group contains 8 animals. Comparisons were made on the basis of the one-way ANOVA followed by Bonferroni multiple tests. All groups were compared to the DMH control group ($^{a}p< 0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$).

Table 7: Effect of taVNS on biological markers of oxidative stress against DMH induced colon carcinogenesis

	Control	**taVNS control**	**DMH control**	**taVNS 1**	**taVNS 2**	**taVNS 3**	**taVNS 4**	**taVNS 5**	**Standard chemotherapy**	**Dummy control**
TBARs (nM of MDA / µg of protein)	101.06±4.81	140±14.90[c]	187.82±6.78	124.35±6.11[c]	104.70±1.95[c]	126.49±17.39[c]	131.75±3.24[c]	124.35±4.50[c]	136.36±22.86[c]	146.98 ±9.81[b]
Glutathione (mg %)	0.36±0.01[c]	0.47±0.02[c]	0.68±0.01	0.61±0.12[c]	0.38±0.01[c]	0.45±0.06[c]	0.32±0.06[c]	0.36±0.01[c]	0.35±0.03[c]	0.38±0.01[c]
Catalase (nM of H_2O_2 /min. /mg of protein)	1.75±0.1	2.18±0.09	1.67±0.09	2.13±0.15[c]	1.81±0.03	2.09±0.03[c]	1.76±0.02	1.83±0.02[b]	2.15±0.08[c]	1.69±0.08
SOD (unit of SOD /mg of protein)	0.03±0.002[c]	0.04±0.002[c]	0.004±0.001	0.02±0.003[c]	0.03±0.001[c]	0.03±0.002[c]	0.02±0.001[c]	0.04±0.002[c]	0.04±0.004[c]	0.02±0.01[c]
Protein carbonyl (nM/ml unit)	34.31±3.74	50.89±8.65[c]	106.68±10.8	53.90±6.02[c]	74.99±3.69[c]	58.78±7.63[c]	102.21±2.59	62.63±3.50[c]	40.30±1.95[c]	44.14±5.45[c]

(Values are presented as Mean ±SD), each group contains 8 animals. Comparisons were made on the basis of the one-way ANOVA followed by Bonferroni multiple tests. All groups were compared to the DMH control group ([a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$

Morphological and histopathological evaluation

The colonic tissue was evaluated morphologically through methylene blue staining, H&E staining and SEM. After methylene blue staining of colonic mucosa of DMH treated animals, dark blue stained cells were visualized representing ACF. The ACF in the colonic mucosa of the DMH treated animals was also evident through H&E staining and SEM. The SEM images of DMH treated animals were also evident for the small neoplastic lesions (Figure XXXIII). taVNS3 and 4 curtailed the ACF formation, normalised the histopathological architecture more favourably. The formation of neoplastic lesions was also subsided after the taVNS application in general and profoundly by taVNS 3 and 4 (Figure 4).

Western blotting

Proteins extracted from individual groups were subjected to immunoblotting assay for anti-apoptotic (Bcl-2 and Bcl-xl), pro-apoptotic (BAX and BAD) proteins along with downstream apoptotic markers (VDAC, cytochrome-c, Apaf-1 and procaspase 9).

After treatment with DMH, the expression of anti-apoptotic proteins (Bcl-2 and Bcl-xl) was increased. The expression of pro-apoptotic protein BAX was decreased while the BAD increased. The taVNS application resulted in downregulation of Bcl-2, with mixed effects on expression of Bcl-xl was noted. taVNS 1, 2 and 3 resulted in Bcl-xl upregulation while taVNS 4 and 5 resulted in downregulation. In response to DMH treatment, expression of BAX decreased while BAD increased. taVNS resulted in downregulation of BAX (except taVNS 5) and upregulation of BAD. All in all taVNS was recorded to have more favourable regulation of pro- and anti-apoptotic proteins, when compared to DMH treated animals.

DMH administration was also evident for increased expression for VDAC, Apaf-1 and procaspase 9 with concomitant decrease in cytochrome c expression.

taVNS down-regulated the VDAC (taVNS1 and 5), Apaf-1 and procaspase-9 (except taVNS 4) expression (Figure 5a). taVNS favourably regulated the expression of VDAC (taVNS 1), Apaf-1 (taVNS 1 and 2) and procaspase-9 (except taVNS 5).

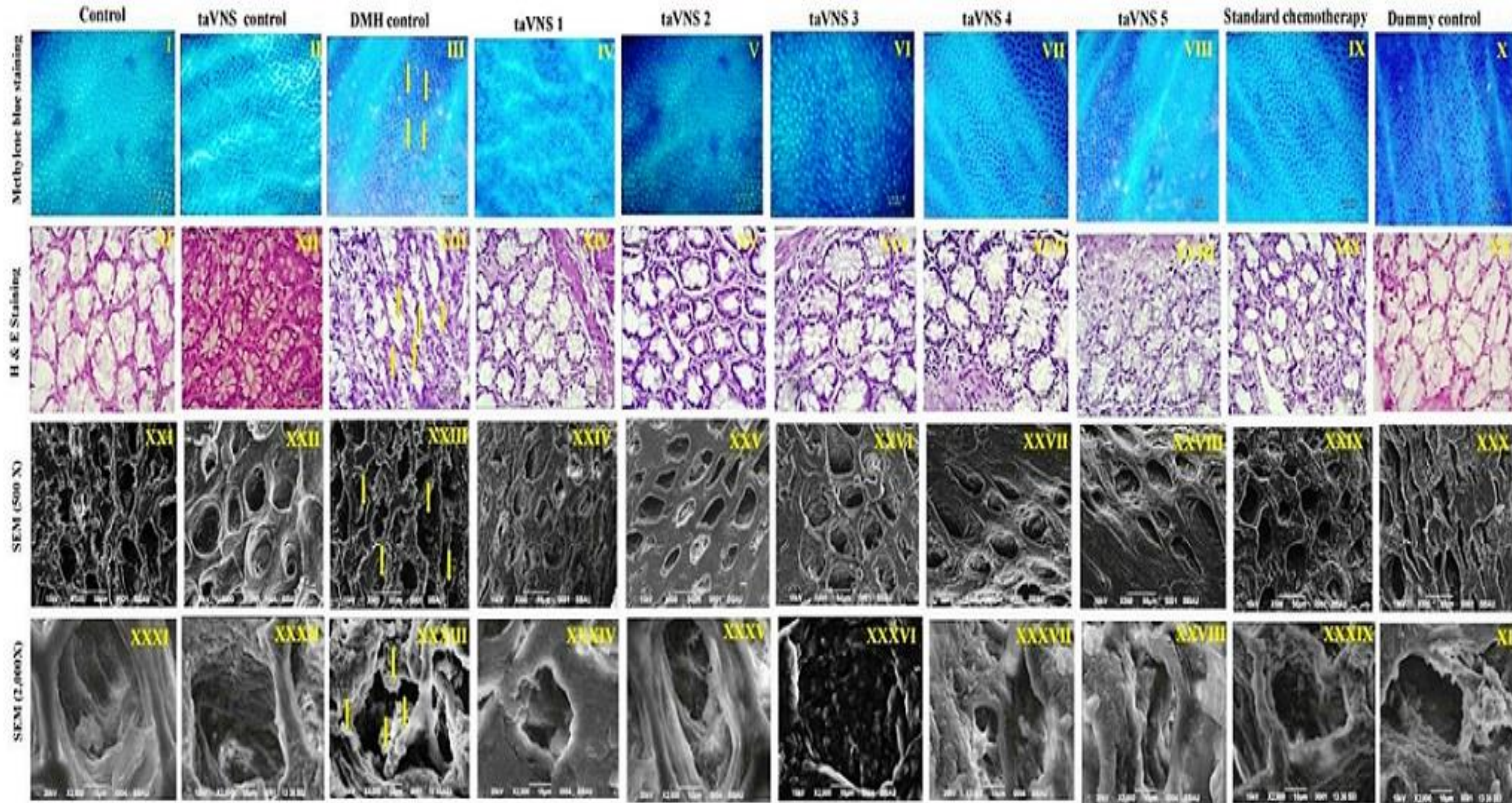
DMH treatment was also evident for decreased levels of caspase 3 and 8, which were up-regulated after taVNS application (Figure 5c and d).

DMH treatment decreased the α7nAchR expression with up-surged expression of NFκB, TNF-α and HMGB -1. Majority of taVNS treatments down-regulated the expression for NFκB (except taVNS3),TNF-α, HMGB-1 and up-regulated the α7nAchR expression (except taVNS 3) (Figure 6a). On considering standard chemotherapy, majority of taVNS treatments resulted in better responses in regulation of CAP associated proteins with exception of α7nAchR.

Quantitative RT-PCR

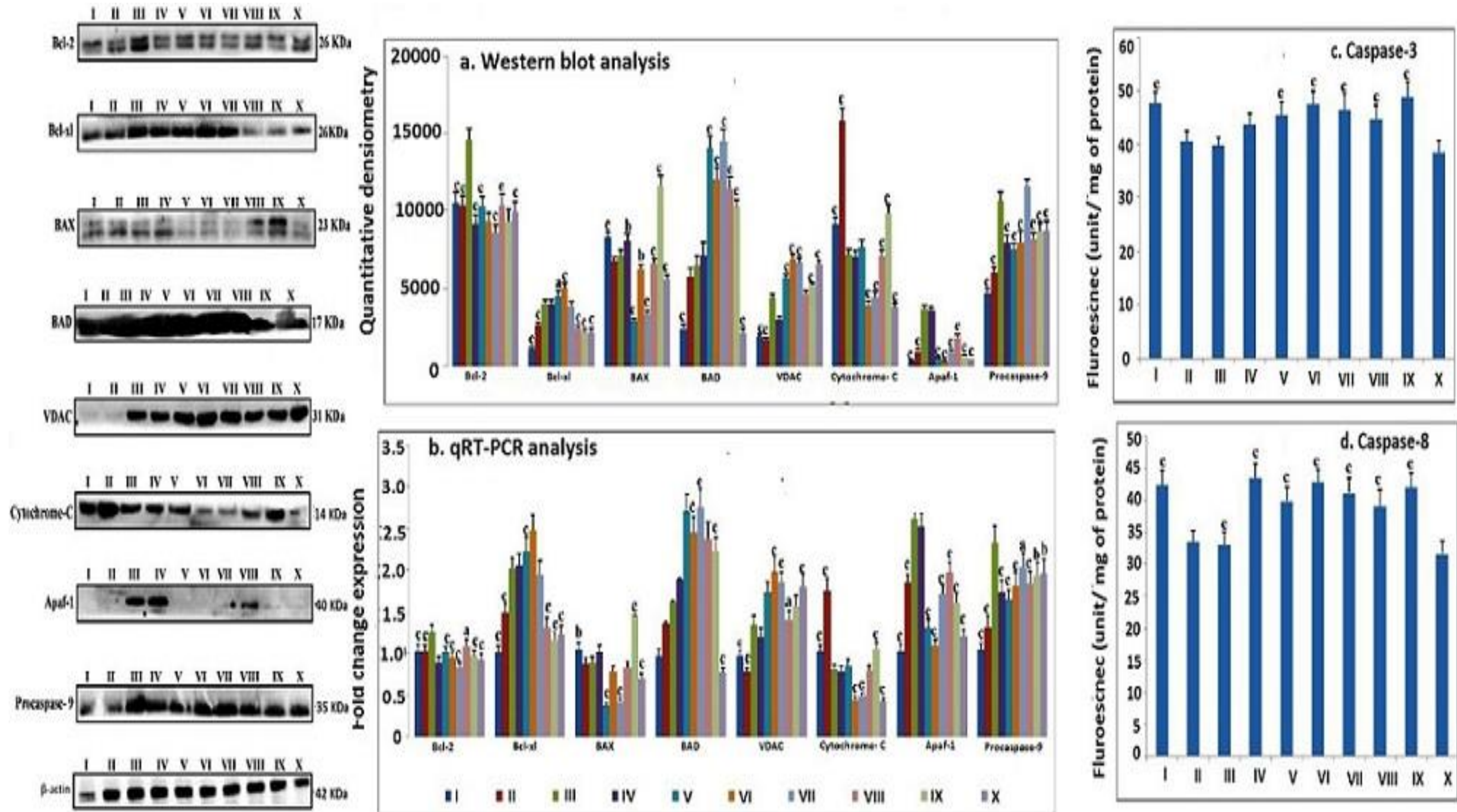
The qRT-PCR assay was used for affirmation of genetic phenotypes for the protein markers associated with mitochondrial apoptotic pathway and CAP as scrutinized through immunoblotting assay. The qRT-PCR assay validated the mRNA expression of proteins scrutinized through immunoblotting (Figure 5b and 6b).

Figure 4: Microscopic evaluation of colon tissue treated with taVNS against DMH induced colon carcinogenesis



I-X:– Methylene blue staining of colon tissue; XI-XX:– represents H & E staining of colon tissue; XXI-XXX:- represents scanning electron microscopy of colon tissue (500X) and XXXI-XL:- represents scanning electron microscopy of colon tissue (2000X) depicts presence of small neoplastic lesions on mucosal tissue of DMH control . The arrows represent ACF formation and neoplastic lesions.

Figure 5: Effect of taVNS on mitochondrial apoptosis against DMH induced colon carcinogenesis



The details of groups as follows for western blot analysis and caspase assay-

I-Control, II-taVNS control, III- DMH control, IV- taVNS1, V-taVNS 2, VI-taVNS 3, VII- taVNS 4, VIII-taVNS 5, IX- Standard chemotherapy, X- Dummy control.

Specific treatments for particular groups mentioned in table 2.

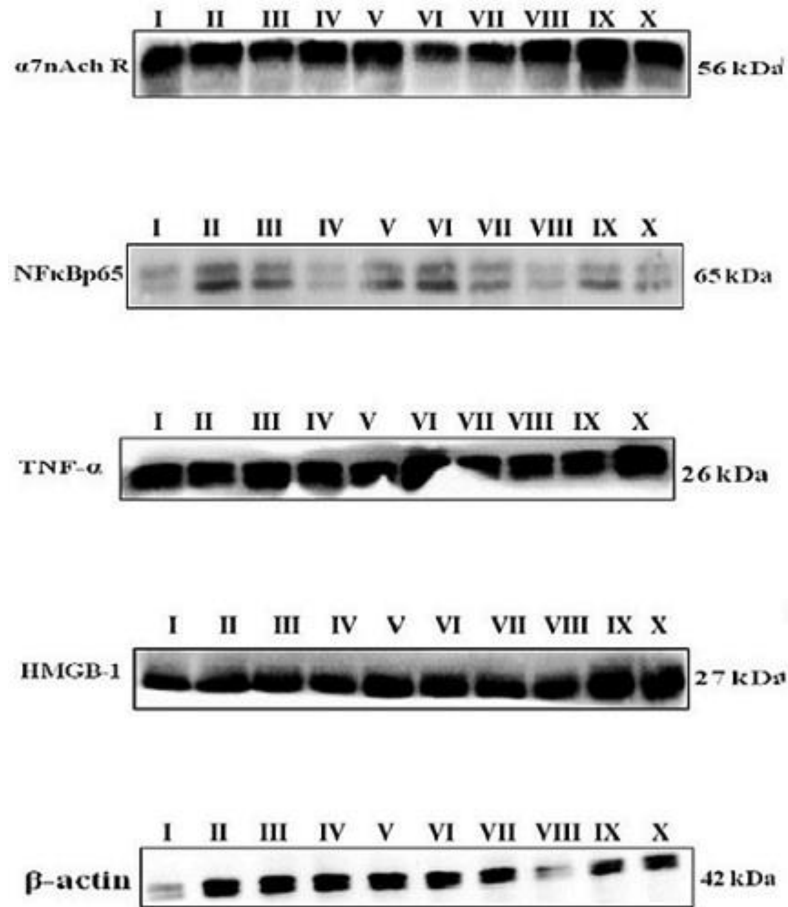
a. Proteins were extracted from individual groups and subjected to immunoblotting of pro-apoptotic (BAX and BAD) and anti-apoptotic (Bcl-2 and Bcl-xl) markers along with downstream apoptotic markers (VDAC, cytochrome-c, Apaf-1 and procaspase 9). After treatment with DMH the anti-apoptotic proteins (Bcl-2 and Bcl-xl) expression was increased. The expression of pro-apoptotic protein BAX decreased while BAD increased. The taVNS treatment results in restoration of anti-apoptotic and pro-apoptotic proteins with exception of BAD. When observed the expression of downstream markers of mitochondrial apoptosis (VADC, cytochrome c, Apaf-1 and procaspase 9), treatment with DMH results in increased expression of VDAC, Apaf-1 and procaspase 9 along with decreased expression of cytochrome c. Treatment with taVNS resulted in increased expression of cytochrome c and decreased expression of procaspase 9.

b. The outcomes from the immunoblotting assay were confirmed through qRT-PCR studies by scrutinizing the respective phenotypes of pathway associated proteins. The mRNA expressions of above mentioned protein were also in line with the findings of immunoblotting assay. β-actin was used as loading control in immunoblotting and qRT-PCR assay.

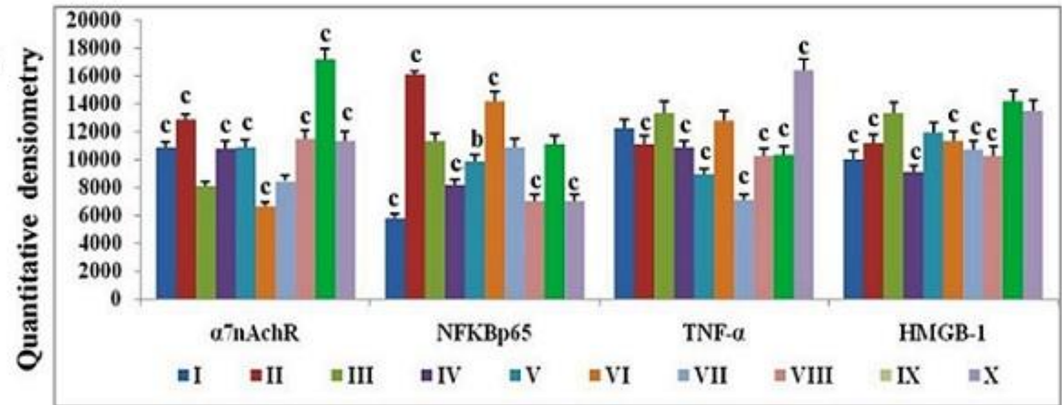
c and d: Represents the levels of caspase 3 and 8 respectively. The activity of caspase was detected by commercial fluorescence based assay. The caspase 3 and caspase 8 levels were decreased after DMH treatment and taVNS (particular taVNS 3) up-regulated the same. Each experiment was performed in triplicate. Values are

presented as mean ± SD. Comparisons were made by the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the DMH treated group ($^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$).

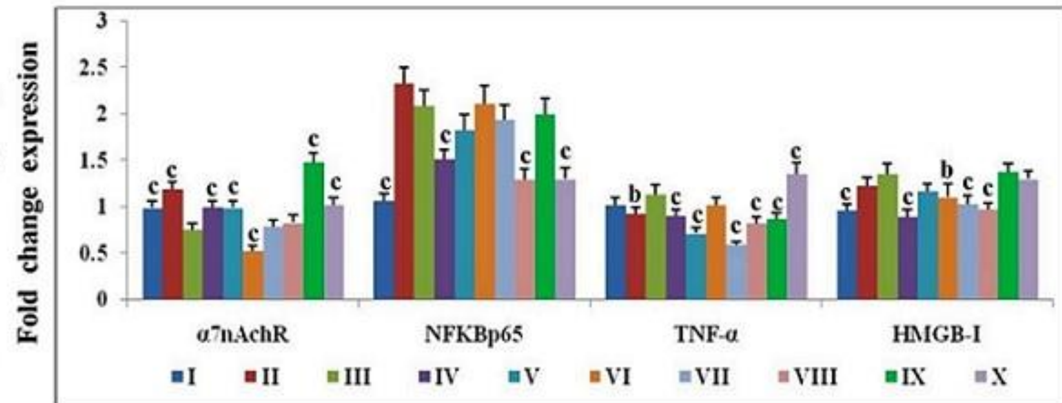
Figure 6: Effect of taVNS on cholinergic anti-inflammatory pathway against DMH induced colon carcinogenesis



a. Western blot analysis



b. qRT-PCR analysis



The details of groups as follows for western blot analysis –

I-Control, II-taVNS control, III- DMH control, IV- taVNS 1, V-taVNS 2, VI-taVNS 3, VII- taVNS 4, VIII-taVNS 5, IX- Standard chemotherapy, X- Dummy control. Specific treatments for individual groups mentioned in table 2.

a. Immunoblotts for α7nAchR, NFκB, TNF-α and HMGB-1 for various groups. The expression of α7nAchR was decreased while NFκB, TNF-α and HMGB-1 increased after DMH treatment. The taVNS treatment resulted in increased expression of α7nAchR and curtailment of inflammatory markers (NFκB, TNF-α and HMGB-1).

b. The mRNA expressions of above mentioned protein were also in line with the findings of immunoblotting assay. β-actin was used as loading control in immunoblottingand qRT-PCR assay. Each experiment was performed in triplicate. Values are presented as mean ± SD. Comparisons were made by the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the DMH treated group ($^{a}p<0.05$,$^{b}p<0.01$, $^{c}p<0.001$).

(b) Effect of α7nAchR agonist alone and in combination with transcutaneous vagus nerve stimulation on mitochondrial apoptosis against 1,2-dimethyl hydrazine induced colon carcinogenesis in *albino wistar* rats

Hemodynamic changes

The treatment with DMH results in notable variations in electrocardiographic parameters (Figure 1a).

The treatment with DMH was very well revealed increment in HR (395.001±10.67bpm), QRS interval(0.03±0.002 s) and Q amplitude (0.17±0.02 s) along with decrement in RR (0.15±0.02s), QT interval (0.05±0.003s) , JT interval (0.04±0.002 s), QTc (0.13±0.01s), P amplitude (0.04±0.01mv), R amplitude (1.28±0.09mv), S amplitude (-0.15±0.01 mv), T amplitude (0.14±0.01) and ST height (0.05±0.002 mv) without affecting PR interval, P duration and T peak and T end interval (Table 8).

On consideration of HRV analysis, a significant decrement noted with median RR (152.8±8.18 ms), average RR (151.89±8.25ms), SDRR (3.87±0.26 ms), CVRR (0.03±0.001ms), LF (0.11±0.00 μs^2), HF (0.91±2.76 μs^2), LF/HF (.12±0.00) and VLF (0.39±0.02 μs^2) with DMH treatment (Table 9).

The PCA and PLS-DA analysis of the ECG and HRV signals demonstrated that treatment with PNU and PNU+ taVNS favourably regulate the ECG and HRV signals towards the normal against DMH induced colon carcinogenesis (Figure 7b).

Table 8: Effect of PNU alone and in combination with taVNS on ECG against DMH induced colon carcinogenesis

ECG Parameters	Control	taVNS control	DMH control	Standard chemotherapy	Dummy control	DMH+PNU	DMH+PNU+VNS
RR Interval (s)	0.18±0.01[c]	0.16±0.01	0.15±0.02	0.16±0.01	0.16±0.01	0.17±0.01	0.17±0.01
HR (BPM)	340.11±12.67[c]	381.92±9.63	395.001±10.67	378.82±23.97	385.25 ±10.66	356.36±19.11[b]	345.63±18.63[c]
PR Interval (s)	0.03±0.001	0.03±0.001	0.03±0.001	0.03±0.001	0.03±0.001	0.03±0.002	0.03±0.002
P Duration (s)	0.02±0.001	0.02±0.002	0.02±0.001	0.02±0.002	0.02±0.001	0.02±0.001	0.02±0.001
QRS Interval (s)	0.02±0.002[c]	0.03±0.002	0.03±0.002	0.03±0.002	0.02±0.002[c]	0.02±0.001[c]	0.02±0.001[c]
QT Interval (s)	0.06±0.003	0.04±0.002	0.05±0.003	0.04±0.003	0.04±0.002	0.04±0.003	0.04±0.002
J T Interval (s)	0.05±0.003[c]	0.02±0.002[c]	0.04±0.002	0.02±0.002[c]	0.02±0.001[c]	0.04±0.003	0.04±0.002
Tpeak T end Interval (s)	0.04±0.003	0.03±0.002[c]	0.04±0.02	0.02±0.002[c]	0.03±0.002[c]	0.03±0.002[c]	0.03±0.001[c]
QTc (s)	0.15±0.01[b]	0.12±0.08	0.13±0.01	0.10±0.07[c]	0.09±0.008[c]	0.10±0.09[c]	0.11±0.08[b]
P Amplitude (mV)	0.10±0.04[c]	0.06±0.02	0.04±0.01	0.05±0.002	0.07±0.01[a]	0.07±0.02[a]	0.04±0.01
Q Amplitude (mV)	0.04±0.002[c]	0.06±0.0004[c]	0.17±0.02	0.02±0.002[c]	0.03±0.004	0.02±0.003[c]	0.04±0.002[c]
R Amplitude (mV)	1.70±0.12[c]	0.92±0.07[c]	1.28±0.09	0.71±0.05[c]	1.12±0.08[a]	1.04±0.08[c]	0.83±0.05[c]
S Amplitude (mV)	-0.13±0.01	-0.09±0.007[c]	-0.15±0.01	-0.11±0.007[c]	-0.05±0.002[c]	-0.16±0.01[c]	-0.36±0.02[c]
T Amplitude (mV)	0.43±0.03[c]	0.05±0. /001[c]	0.14±0.01	0.08±0.002[c]	0.19±0.01[c]	0.28±0.02[c]	0.09±0.001[c]
ST Height (mV)	0.10±0.02[c]	0.08±0.01[c]	0.05±0.002	0.06±0.003[c]	0.04±0.001[c]	0.05±0.002	0.05±0.003

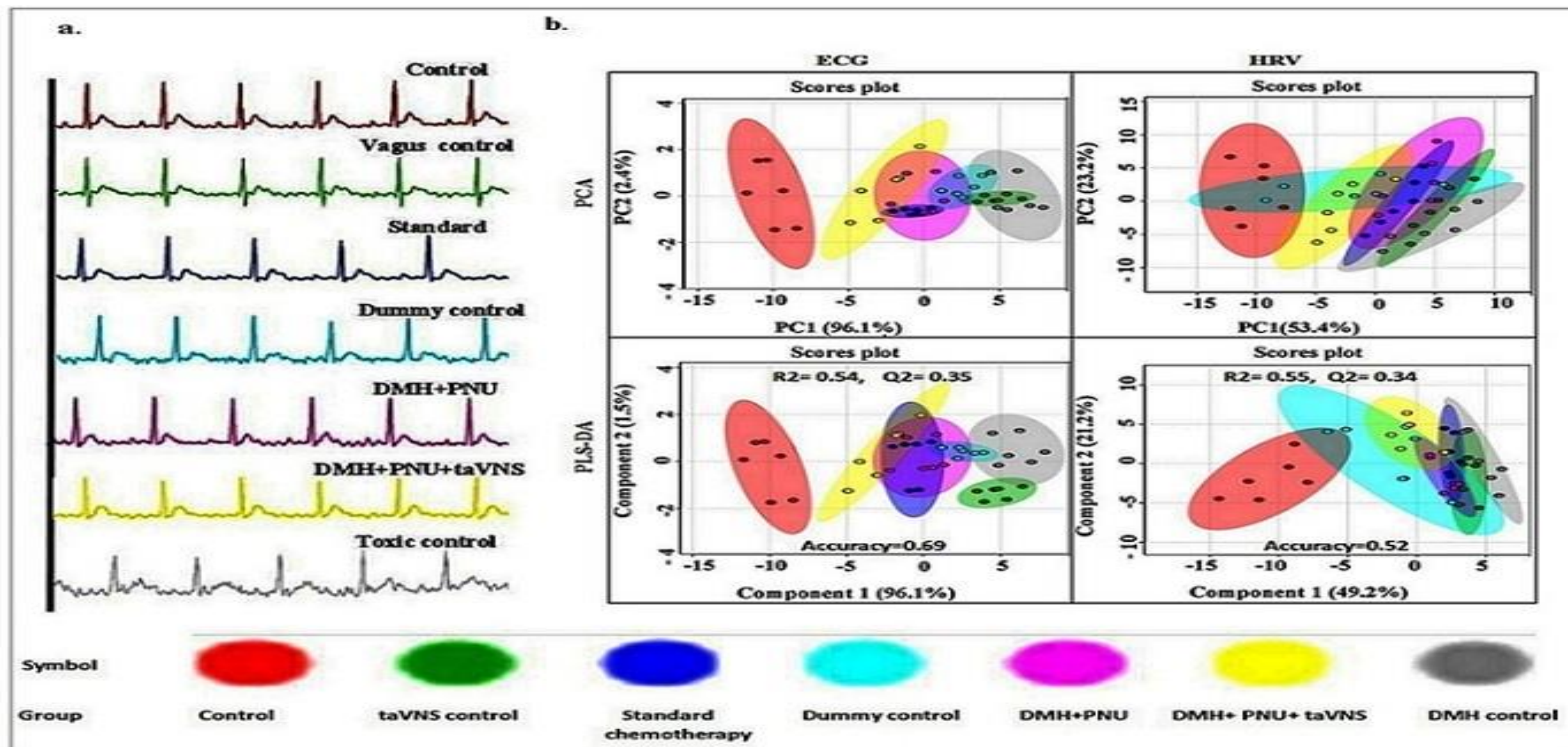
(Values are presented as Mean ±SD), each group contains 8 animals. Comparisons were made on the basis of the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the DMH control group ([a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$

Table 9: Effect of PNU alone and in combination with taVNS on HRV against DMH induced colon carcinogenesis

HRV Parameters	Control	taVNS control	DMH control	Standard chemotherapy	Dummy control	DMH+PNU	DMH+PNU+taVNS
Time domain							
Average RR(ms)	176.41±8.77[c]	157.10±4.24	151.89±8.25	158.38±5.32	155.74±3.21	168.36±6.34[b]	173. 59±7.56[c]
Median RR (ms)	177.00±0.78[c]	158.6±6.34	152.8±8.18	159.8±4.45	156.00±5.45[b]	170.20±14.54[a]	176.14±9.76[cs]
SD RR (ms)	6.43±0.53[c]	9.28±0.64[c]	3.87±0.26	7.30±0.54[c]	3.16±0.23	9.13±0.76[c]	3.23±0.23
SDARR	3.30±0.23[c]	8.31±0.67[c]	8.745±3.45	7.02±6.75[c]	7.2±0.13[c]	6.11±0.43	3.25±0.26[c]
CVRR (ms)	0.04±0.001[c]	0.06±0.003[c]	0.03±0.001	0.04±0.002[c]	0.03±0.00	0.06±0.002[c]	0.03±0.003
Frequency domain							
LF(μs^2)	16.18±0.96[c]	0.12±0.00	0.11±0.00	0.59±0.05	1.02±0.02	4.12±0.37[c]	0.07±0.00
HF(μs^2)	65.64±3.76[c]	0.64±0.04[c]	0.91±2.76	5.13±0.41[c]	6.38±0.52[c]	15.63±0.94[c]	0.32±0.026[c]
LF/HF	0.24±0.018[c]	0.18±0.01[c]	0.12±0.00	0.11±0.00[c]	0.16±0.00	0.26±0.12[c]	0.21±0.00[c]
VLF (μs^2)	1.57±0.12[c]	2.34±0.16[c]	0.39±0.02	1.61±0.13[c]	2.25±0.14[c]	3.32±0.27[c]	1.45±0.03

(Values are presented as Mean ±SD), each group contains 8 animals. Comparisons were made on the basis of the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the DMH control group ([a]$p< 0.05$, [b]$p< 0.01$, [c]$p< 0.001$).

Figure 7: Represenataive ECG recordings and analysis in response to against taVNS against DMH induced colon carcinogenesis



- **a.** Representative ECG recordings of control, DMH and taVNS treatments.
- **b.** The figure represents for 2D score plots derived from PCA and PLS-DA based analysis for ECG and HRV signals in different groups.

pH, total acidity, percentage weight variation and ACF count

On consideration for physiological parameters, significant decrease in weight (-12.33±0.82 %), intestinal pH (6.38±0.18) with significant increase in total acidity (126.86±5.26 $mEql^{-1}$) and ACF (63.92±1.87 NoS) was noted with DMH treatment. Treatment with PNU + taVNS significantly regulate above mentioned parameters towards normal control group in compare to DMH+PNU (Table 10).

Table 10: Effect of PNU alone and in combination with taVNS on physiological parameters against DMH induced colon carcinogenesis

	Weight variation (%)	pH	Total acidity ($mEql^{-1}$)	Aberrant crypt (NoS)
Control	18.82±1.15^{c}	6.97±0.31^{b}	94.15±2.15^{c}	13.76±0.97^{c}
taVNS control	17.31±1.13^{c}	6.97±0.25^{b}	91.56±3.6^{c}	15.10±0.87^{c}
DMH control	-12.33±0.82	6.38±0.18	126.86±5.26	63.92±1.87^{c}
Standard chemotherapy	11.84±0.98^{c}	7.01±0.21^{b}	93.17±2.12^{c}	15.27±0.95^{c}
Dummy control	22.52±1.12^{c}	6.94±0.23^{b}	93.13±4.23^{c}	19.62±0.76^{c}
DMH+ PNU	17.76±0.75^{c}	6.98±0.22^{b}	99.34±3.13^{c}	16.18±0.69^{c}
DMH+PNU+ taVNS	20.76±1.04^{c}	6.96±0.23^{b}	94.38±3.10^{c}	14.21±0.88^{c}

(Values are presented as Mean ± SD), each group contains 8 animals. Comparisons were made on the basis of the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the DMH control group ($^{a}p< 0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$).

Antioxidant assay

When accounted for biochemical evaluation, there was momentous increase in TBAR's (193.32±6.78nM of MDA /µg of protein) and PC (102.18±4.82nM/ml unit) was noted in DMH treated animals in collation to control, indication of increase oxidative stress. Treatment with PNU+taVNS more effectively restores the levels of same towards normal in compare to PNU treatment alone. DMH treatment was noted with upsurge in tissue glutathione (0.72±0.01 mg %) level and decrease in levels of

SOD (0.005±0.001unit of SOD /mg of protein) and catalase (1.60±0.09 nM of H_2O_2 /min. /mg of protein). Treatment with PNU and PNU +DMH both significantly restores the enzymatic activity of glutathione, SOD and catalase but treatment with PNU+DMH was comparatively more effective (Table 11).

Table 11: Effect of PNU alone and in combination with taVNS on biological markers of oxidative stress against DMH induced colon carcinogenesis

	Control	taVNS control	DMH control	Standard chemotherapy	Dummy control	DMH+ PNU	DMU+PNU+ taVNS
TBARs (nM of M DA /µg of protein)	105.16±4.81^c	145±9.90^c	193.32±6.78	141.16±7.22^c	152.18±9.81^c	144.10±2.99^c	134.96±4.32^c
Glutathione (mg %)	0.39±0.01^c	0.50±0.02^c	0.72±0.01	0.38±0.02^c	0.41±0.01^c	0.40±0.03^c	0.41±0.03
Catalase(nM of H_2O_2 /min. /mg of protein	1.70±0.1	2.13±0.09	1.60±0.09	2.08±0.08^c	1.60±0.08	1.98±0.11	2.47 ±0.08
SOD(unit of SOD /mg of protein)	0.04±0.002^c	0.05±0.002^c	0.005±0.001	0.05±0.004^c	0.03±0.01^c	0.03± 0.001^c	0.05 ± 0.003^c
Protein carbonyl(nM/ml unit)	33.22±1.74^c	49.19±3.15^c	102.18±4.82	42.100±1.95^c	43.23±2.15^c	81.751±6.30^c	41.84±1.76^c

(Values are presented as Mean ± SD), each group contains 8 animals. Comparisons were made on the basis of the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the DMH control group ($^a p < 0.05$, $^b p < 0.01$, $^c p < 0.001$).

Morphological and histopathological evaluation

Morphologically colon tissues were examined through methylene blue staining, H&E staining and SEM. Notable alterations in morphology was observed in morphological examinations.

Presence of dark blue stained cells after methylene blue staining were representing the ACF in DMH treated animals which also evident through SEM and H&E staining. Small neoplastic lesions were visualized in SEM images of DMH treated colon tissue (Figure 8-XXIV). A treatment with PNU and PNU+ taVNS both diminishes the ACF formation and restores the normal architecture of colon mucosa. Along with above protective effects neoplastic lesions formation was diminished with PNU and PNU+taVNS treatment but in particular PNU +taVNS treatment was comparatively more effective in restoration of normal architecture of colon mucosa.

Western blotting

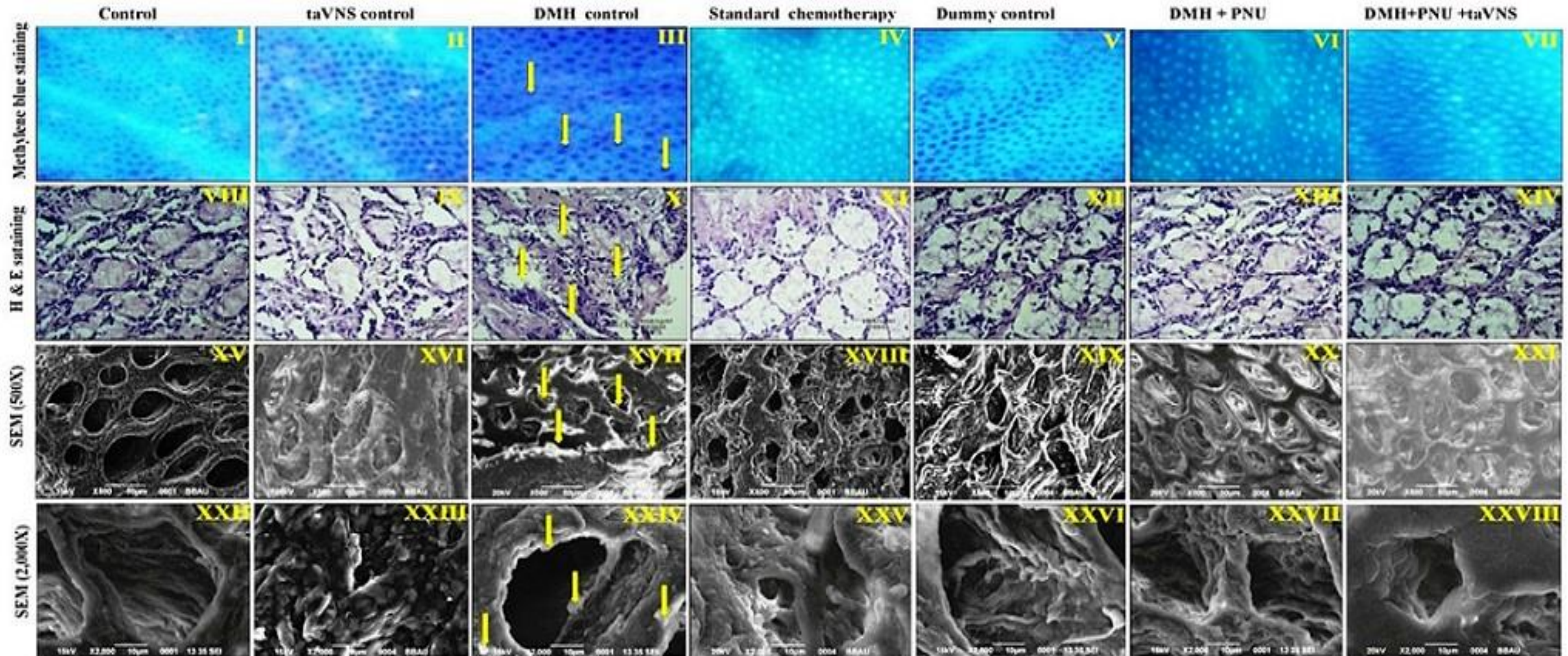
Immunoblotting assays were performed to study the effect of treatments on expression of proteins in associated pathways. After immunoblotting study, notable variations were observed in expression of proteins. The expression of anti-apoptotic proteins (Bcl-2 and Bcl-xl) was increased while pro-apoptotic protein (BAX and BAD) expression reduced in response to DMH treatment. The treatment with PNU and PNU + taVNS resulted in downregulation of anti-apoptotic proteins and upregulation of pro-apoptotic proteins.

When accounting downstream markers of mitochondria mediated apoptotic pathway, the DMH administration results in downregulation of Cytochrome expression with simultaneous upregulation of VDAC, Apaf-1and procaspase 9. The treatment with PNU and PNU + taVNS favours the mitochondrial apoptotic pathway by regulating the expression of linked proteins (Figure 9 a).

In response to DMH treatment, the expression of NFκB and UCHL -1 up regulated. The expression of both NFκB and UCHL -1 down regulated in response to PNU and PNU +taVNS treatment (Figure 9 b).

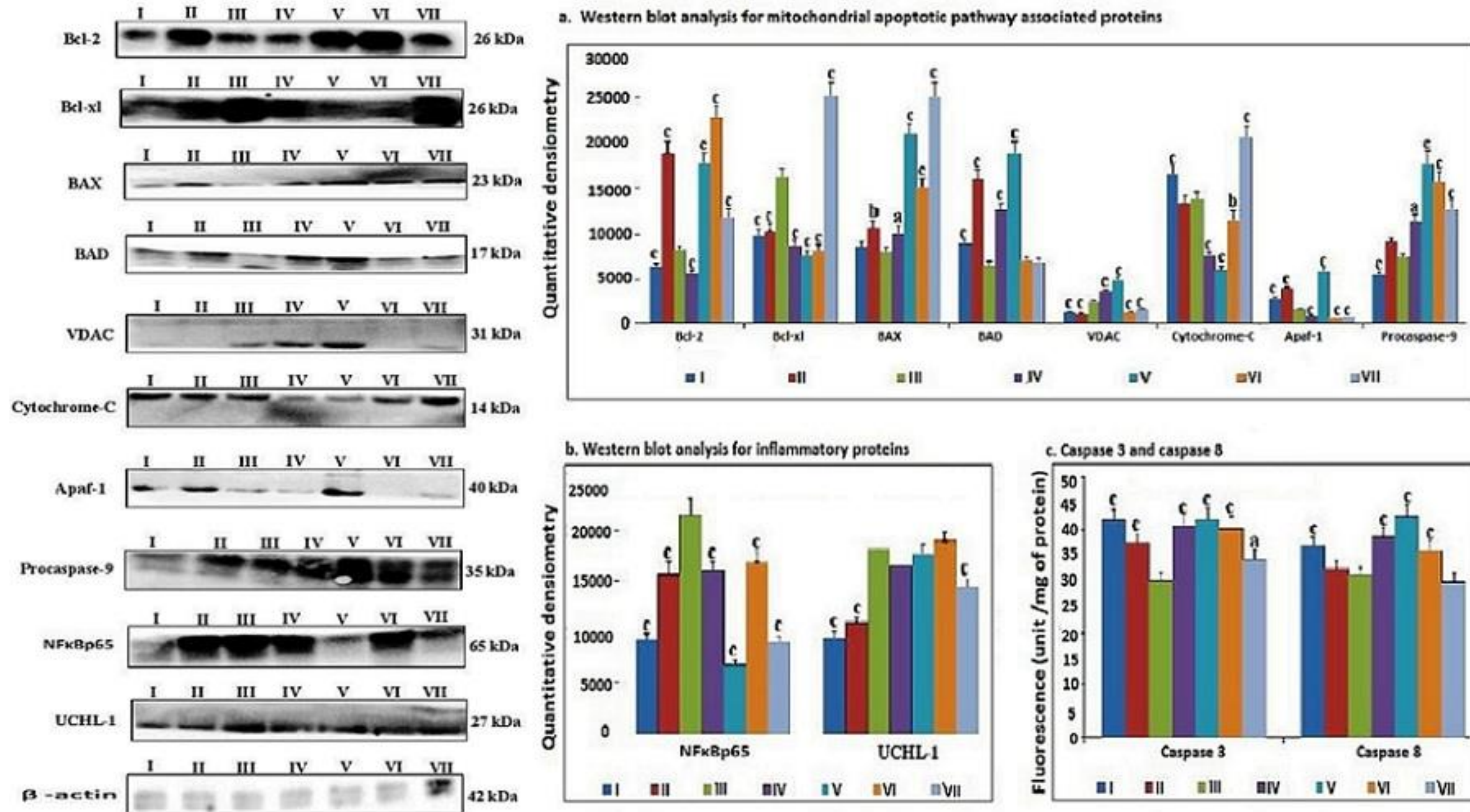
The expression of caspase 3 and caspase 8 was down regulated with DMH treatment, restored on PNU and PNU + taVNS application (Figure 9c).

Figure 8: Microscopic evaluation of colon tissue treated with PNU and taVNS against DMH induced colon carcinogenesis



I-VII:– Methylene blue staining of colon tissue; VIII-XIV:– represents H & E staining of colon tissue; XV-XXI:- represents scanning electron microscopy of colon tissue (500X) and XXII-XXVIII :- represents scanning electron microscopy of colon tissue (2000X) depicts presence of small neoplastic lesions on mucosal tissue of DMH control . The arrows represent ACF formation and neoplastic lesions.

Figure 9: Effect of PNU and taVNS on mitochondrial apoptosis and inflammatory proteins against DMH induced colon carcinogenesis



After treatment with DMH the anti-apoptotic proteins (Bcl-2 and Bcl-xl) expression was increased. The expression of pro-apoptotic protein BAX decreased while BAD increased. The taVNS treatment results in restoration of anti-apoptotic and pro-apoptotic proteins with exception of BAD. When observed the expression of downstream markers of mitochondrial apoptosis (VADC, cytochrome c, Apaf-1 and procaspase 9), treatment with DMH results in increased expression of VDAC, Apaf-1 and procaspase 9 along with decreased expression of cytochrome c. The treatment with PNU and PNU+ taVNS results in increased expression of cytochrome c and decreased expression of procaspase but treatment with PNU + taVNS was comparatively more effective than other treatments.

b: Effect of PNU+taVNS on inflammatory proteins markers against DMH induced colon carcinogenesis. : Immunoblotting for inflammatory markers performed by following similar method as for mitochondrial apoptotic pathway markers. In response to DMH treatment, expression of inflammatory markers increased which were restored towards normal on PNU and PNU+taVNS treatment.

c: Caspase 3 and caspase 8 assay : The activity of caspase was detected by commercial fluorescence based assay. The caspase 3 and caspase 8 levels was decreased after DMH treatment and treatment with PNU, PNU+ taVNS up regulated the same**.** Treatment with PNU+taVNS comparatively more effective than other treatments of study**.** Each experiment was performed in triplicate. Values are presented as mean ± SD. Comparisons were made by the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the DMH treated group ($^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$).

Effects of PNU on metabolomics profiling against DMH induced colon carcinogenesis

1H-NMR method for serum metabolites profiling

Spectral annotation

As shown in (Figure 10) the representative 1D ^{1}H CPMG NMR spectra from each group were assigned for metabolite signals by the collective use of Chenomx NMR suite (Chenomx Inc., Edmonton, AB, Canada) and data available in databases like Biological Magnetic Resonance Data Bank (BMRB) (Ulrich et al. 2008), The Human Metabolome Database (HMDB) (Wishart et al. 2007) and Madison Metabolomics Consortium Database (MMCD) (Cui et al. 2008). The residual peaks in the spectra were assigned by comparing them with the chemical shifts available using previously reported NMR assignments of metabolites.

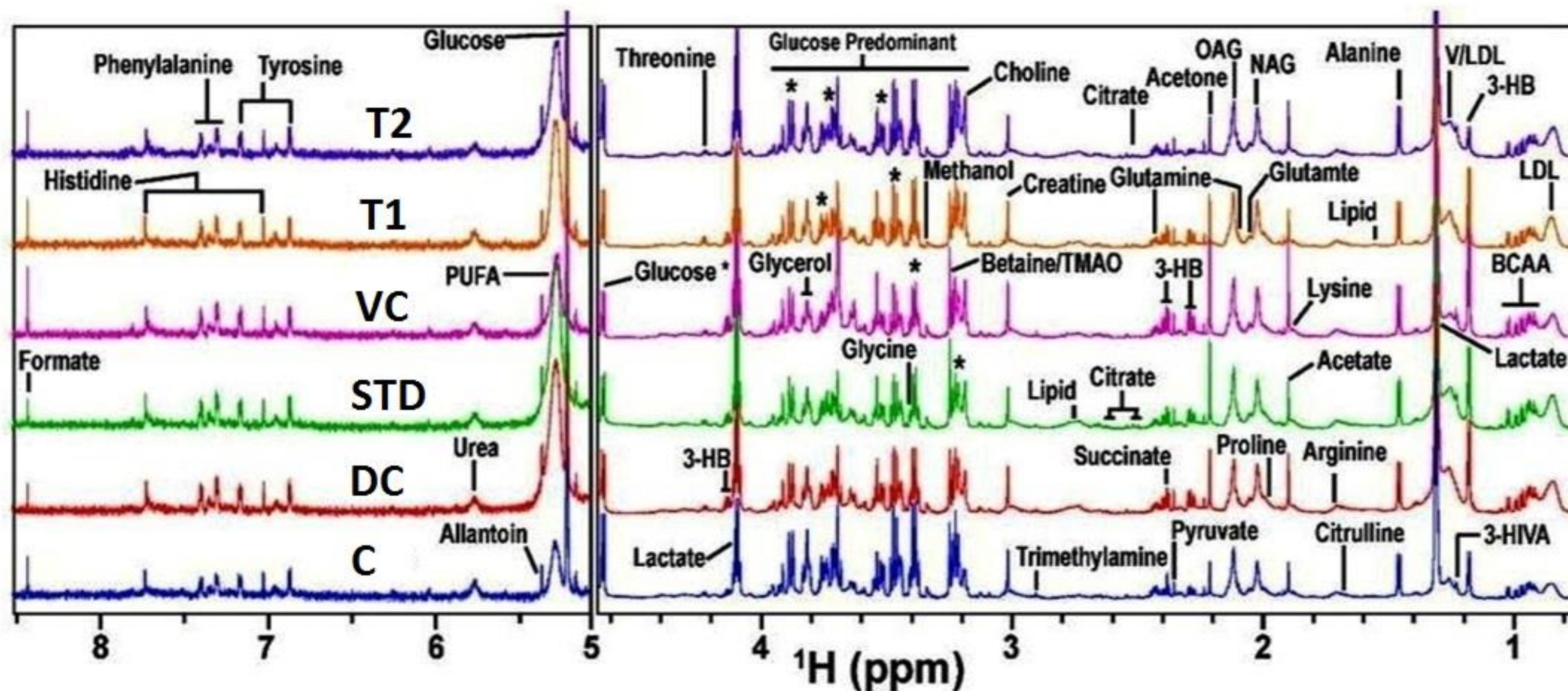
Multivariate data analysis

Multivariate statistical analysis was performed to reduce the dimensionality and complexity of the NMR data and to aid data visualization, clustering and infer the variables that maximize the discrimination between sample groups (Krumsiek and Theis 2013). For each data set, NMR variables were Pareto scaled and subjected to multivariate and univariate statistical analysis in Metabo Analyst. The unsupervised PCA was initially used to analyze the NMR spectral data and to identify general trends and outliers in the samples (Figure 11a).The PCA itself was able to present with a good class separation and clustering, toxic being the farthest and PNU + taVNS group being the closest to normal control group followed by PNU, and standard chemotherapy overlapped with the toxic depicting metabolic similarity, whereas taVNS control formed an independent cluster. Next, the supervised PLS/OPLS -DA analysis wasfurther performed to refine class sepration, better visualization, and

obtain a more accurate statistical analysisas shown in (Figure 11b and 11c) respectively. The PLS-DA and OPLS-DA model validation was performed using 10-fold cross-validationand 100 permutation sshown in their respective 2D score plots. As evident, thecumulative PLS/OPLS-DA score plot revealed that the treatments-PNU and PNU+taVNS are mitigating the effect of toxic as inferred by their progressive shift towards the control group (Figure 12). Likewise, a pair wise PCA, PLS-DA, and OPLS-DA analyses were also performed comparing all the study groups DMH control, standard chemotherapy, taVNS control, PNU, and PNU +taVNS, with respect to the control group (Figure 13). Each pair wise model revealed that there are significant metabolic differences in study groups compared to control animals as evident from the model cross-validation parameters shown in their respective score plots. Further, metabolites responsible for class separation were identified by theirVIP (≥1)scores using PLS-DA model for each group anent to the control.The metabolites responsible for discriminating between the metabolic profiles of toxic and taVNS control in reference to control were considered to study the ameliorating effects of the treatment PNU and PNU+taVNS. As enlisted in Table 12, 23 metabolites were found to be significantly perturbed in the sera of DMH control compared to the control. The serum metabolic profle of DMH treated animals had (a) elevated levels of VLDL/LDL lipoprotein, lipids, PUFA, lactate, NAG, 3-HB, 3-HIVA, acetate, allantoin, BCAA (isoleucine, leucine, valine), AAA (tyrosine, phenylalanine, and histidine) and proline. By the (b) decreased levels of glucose, choline/GPC, glycerol, creatine/creatinine, formate, glycine, and glutamine were observed. The DMH and standard chemotherapy followed a similar pattern of metabolic perturbation expect choline compared to control. Further, the observed

metabolic alterations in DMH control group were ameliorated afterthe treatment PNU and PNU+taVNS as evident from the box plot (Figure 14).

Figure 10: Stack plot of representative 1D ^{1}H NMR spectra of rat sera obtained from different groups (control, DMH and PNU)

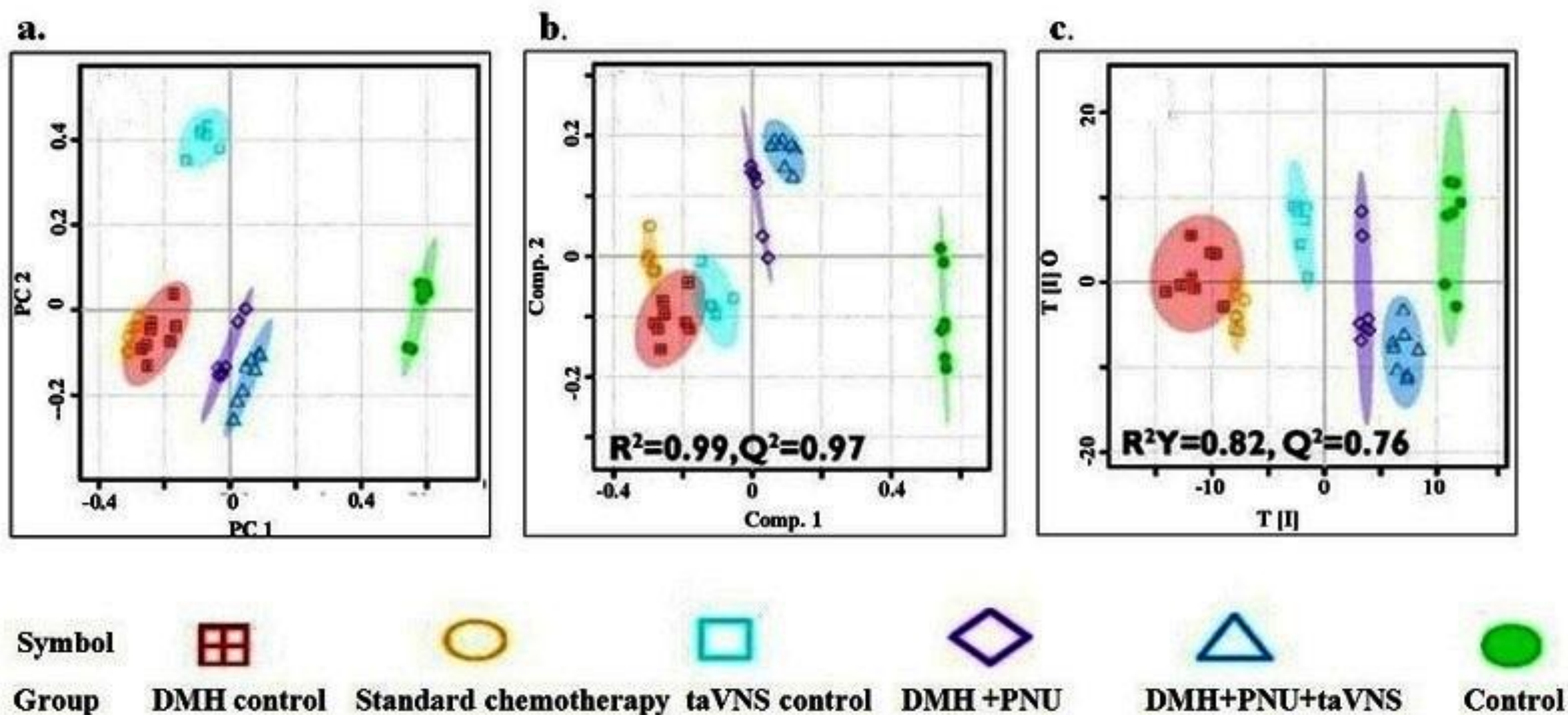


Shows the stack plot of ^{1}H NMR CPMG spectra of serum from each group.

C**: Control , DC: DMH control , STD: Standard chemotherapy , T1: DMH + PNU, T2: DMH+PNU+taVNS**.

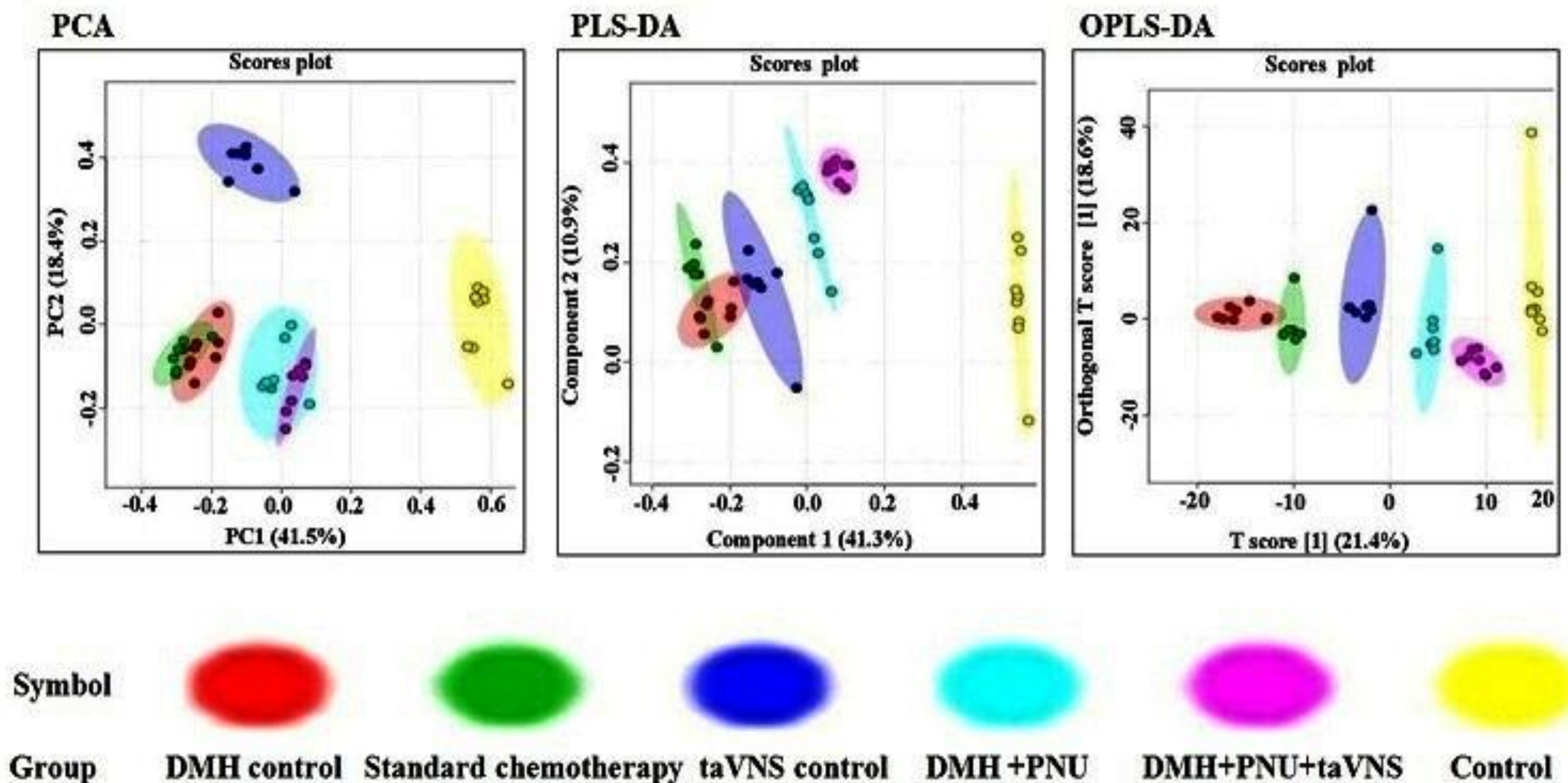
Spectra showed the presence of metabolites including amino acids like alanine, lysine, glutamine, glutamate, branched-chain amino acids (BCAA- isoleucine, leucine, and valine,), aromatic amino acids (AAA- phenylalanine, tyrosine, histidine,) lactate, N-acetyl glycoproteins (NAG), glucose, lipids, creatine, creatinine, acetate, succinate, pyruvate, citrate and lipoproteins (LDL and VLDL), 3-Hydroxyisovalerate (3-HIVA),3-Hydroxybutyrate(3-HB),etc

Figure 11: Multivariate statistical analysis of 1D ^{1}H CPMG NMR spectra of rat sera with DMH , PNU and taVNS treatment



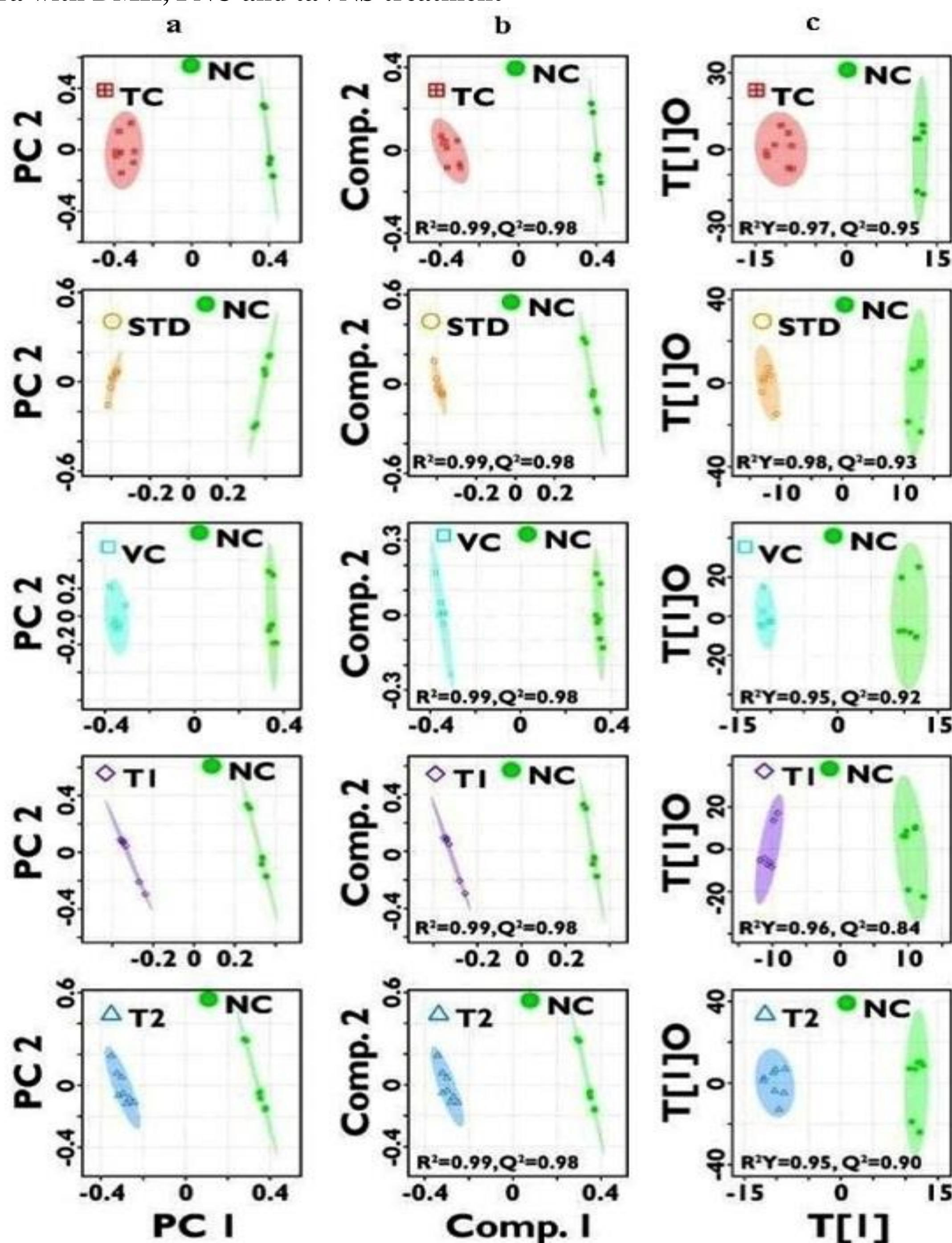
It shows 2D score plots derived the multivariate statistical analysis of 1D ^{1}H CPMG NMR spectra comprising of all the groups. As evident from (a) PCA, (b) PLS-DA, (c) OPLS-DA, the models were able to refine the class separation based on their overall metabolic similarity or dissimilarity to C or DC. Colour circles indicate the 95% confidence interval for each class.

Figure 12: PCA, PLS-DA and OPLS-DA based Multivariate discriminatory analysis for ^{1}HNMR of rat sera with DMH, PNU and taVNS treatment



In figure represents for 2D score plots derived from PCA, PLS-DA and OPLS-DA based analysis for 1HNMR signals in different groups.

Figure: 13 The pair wise 2D- PCA, PLS-DA, and OPLS-DA score plots for rat sera with DMH, PNU and taVNS treatment



NC: Control, VC: taVNS control, DC: DMH control, T1: DMH+PNU, T2: DMH+PNU+taVNS

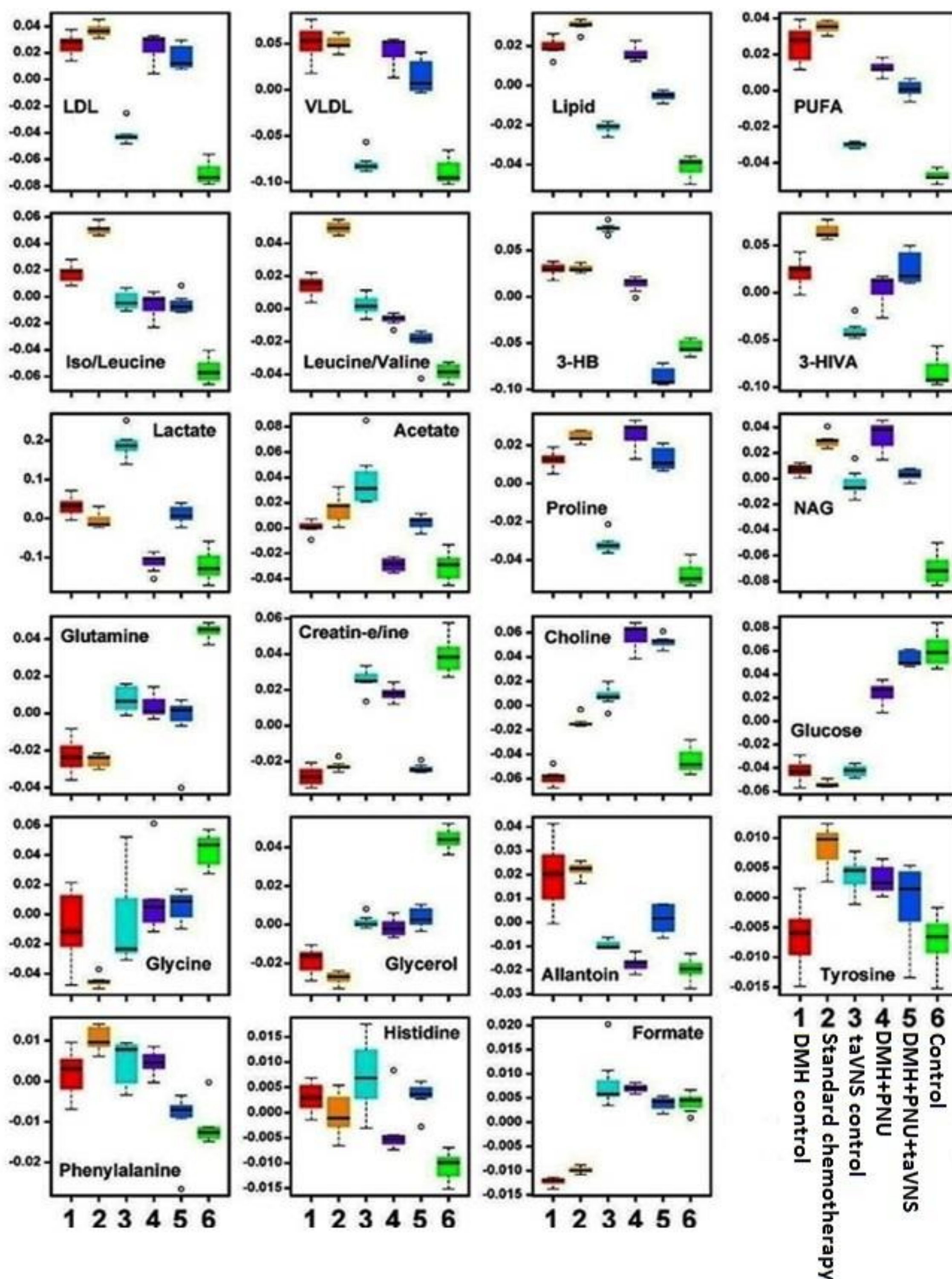
The pair wise 2D- PCA, PLS-DA, and OPLS-DA score plots with their respective R^2, Q^2 values and R^2Y, Q^2values, derived from 1D ^1H CPMG NMR spectra. (a) PCA, (b) PLS-DA (c) OPLS-DA. Coloured circles indicate the 95% confidence interval for each class.

Table 12: Metabolic variabilities among the groups treated with DMH, PNU and combination of PNU and taVNS when compared to normal control

S.no.	^{1}H(ppm)	Metabolites	Normal Control vs.				
			DMH control	Standard chemotherapy	taVNS control	DMH+PNU	DMH+PNU+taVNS
1	0.85	LDL	(↑)	(↑)	(↑)	(↑↑)	(↑↑↑)
2	1.27	VLDL	(↑)	(↑)	(↑)*	(↑↑)	(↑↑↑)
3	0.93	Iso/leucine	(↑)	(↑)	(↑)	(↑↑)	(↑↑↑)
4	0.95	Leucine/Valine	(↑)	(↑)	#(↑)	#(↑↑)	#(↑↑↑)
5	1.19	3-Hydroxybutyrate	(↑)	(↑)	(↑)	(↑)	(↓↓↓)
6	1.23	3-Hydroxyisovalerate	(↑)	(↑)	(↑)	(↑↑↑)	(↑↑)
7	1.31	Lactate	(↑)	(↑)	(↑)	#(↑↑↑)	(↑↑)
8	1.89	Acetate	#(↑)	#(↑)	(↑)	(↑↑↑)	#(↑↑)
9	1.99	Proline	(↑)	(↑)	#(↑)	(↑↑)	(↑↑↑)
10	2.01	NAG	(↑)	(↑)	(↑)	(↑↑)	(↑↑↑)
11	2.43	Glutamine	(↓)	(↓)	#(↓)	(↓↓)	(↓↓↓)
12	2.75	Lipid	(↑)	(↑)	#(↑)	(↑↑)	(↑↑↑)
13	3.01	Creatine/Creatinine	(↓)	(↓)	#(↓)	#(↓↓↓)	(↓)
14	3.19	Choline	#(↓)	#(↑)	(↑)	(↑↑)	(↑↑↑)
15	3.23/3.81	Glucose	(↓)	(↓)	(↓)	(↓↓)	(↓↓↓)
16	3.53	Glycine	(↓)	(↓)	#(↓)	#(↓↓)	(↓↓↓)
17	3.77	Glycerol	(↓)	(↓)	#(↓)	(↓↓)	(↓↓↓)
18	5.27	PUFA	(↑)	(↑)	(↑)	(↑↑)	(↑↑↑)
19	5.37	Allantoin	(↑)	(↑)	(↑)	#(↑↑↑)	(↑↑)
20	6.87	Tyrosine	#(↑)	#(↑)	(↑)	#(↑↑)	(↑↑↑)
21	7.31	Phenylalanine	#(↑)	(↑)	(↑)	(↑↑)	#(↑↑↑)
22	7.71	Histidine	#(↑)	#(↑)	(↑)	#(↑↑↑)	(↑↑)
23	8.43	Formate	(↓)	#(↓)	#(↑)	#(↑↑)	#(↑↑↑)

The perturbed metabolites were sorted based on their VIP Score estimated from the PLS-DA model, for the discriminant significance and p-value < 0.05 for statistical significance. The up (↑) and down (↓) arrows represent the increased and decreased levels of the metabolite in respect to NC. A ↑↑↑/↓↓↓and↑↑/↓↓ score were given to the metaboliteswhich showed ameliorating effects from TC toward NC.

Figure 14: Box-cum-whisker plot of representative 1D ^{1}H NMR spectra of rat sera with DMH, PNU and taVNS treatment



Shows th-e Box and Whisker plots of metabolites found perturbed as listed in Table 3 to aid in visualization of results for following treatment schedules.

Discussion:

(a)Transcutaneous vagus nerve stimulation regulates cholinergic anti-inflammatory pathway to counteract 1, 2-dimethyl hydrazine induced colon carcinogenesis in *albino wistar* rats.

Autonomic dysfunction (decreased HRV) is a well reported phenomenon in clinical survivors of cancer (Palma et al. 2016; Kloter et al. 2018). Previous studies have also endorsed distorted ECG and lower HRV in clinical cases and preclinical cancer models, which was very well apparent in DMH treated animals (Guo et al. 2013; Sammi et al. 2018). taVNS3 was recorded to have most significant effect upon the ECG among all the stimulation methods used. taVNS 3 also up- regulated the time and frequency domain parameters for HRV. It would be appropriate to remark that decrease in HRV has emerged as a non-invasive marker for autonomic dysfunction in cancer survivors (Palma et al. 2016; Kloter et al. 2018; De Couck M 2013). In the instat study taVNS 3 was recorded to regulate autonomic dysfunction more favourably in comparison to other stimulation methods used in the study. taVNS 3 was also recorded to favourably regulate pH, total acidity; weight variation in the DMH treated animals.

Cancer development is a multistep process. Excessive oxidative stress is involved in all stages of cancer development (Skrzydlewska et al. 2001). Increased levels of TBAR's, PC and GSH along with reduced levels of catalase and SOD were noted in response to DMH treatment.

TBAR's and PC are the sensitive and reactive markers for membrane damage and are stable products of lipid and protein peroxidation (Shukla et al. 2014). In present study, significant increase in TBAR's and PC level of DMH treated animals was recorded, supporting the involvement of reactive oxygen species (ROS) in progression of colon cancer. taVNS 2 and 3 resulted, significant reduction in lipid and

protein peroxidation as confirmed by decrease in TBAR's and PC. SOD and catalase are the endogenous free radical scavengers. It would be appropriate to mention that both catalase and SOD work in tandem and forms a defensive team, for protection against injurious free radicals. SOD scavenges superoxide radicals to yield hydrogen peroxide (H_2O_2) and molecular oxygen with subsequent catabolism of H_2O_2 by catalase to water and molecular oxygen (Kaithwas, Dubey, and Pillai 2011; Singh et al. 2008). The decreased levels of SOD and catalase are reported and were recorded with DMH treatment in present study. This decrease in enzymatic activity of SOD and catalase could be attributed to increased utilization in response to oxidative stress. taVNS 2 and 3 resulted in restoration of the SOD and catalase, confirming the say of taVNS in colon carcinogenesis.

Morphological characterization is a well established and affirmative method for cancer diagnosis (Shia et al. 2017). When studied morphologically, the colonic mucosa of the DMH treated animals was conspicuous for presence of ACF. The presence of ACF was very well validated using methylene blue staining, SEM and H&E staining and is in line with previous literature (Mishra et al. 2016).When observed more closely, small neoplastic lesions were also perceived in the colonic tissue. However, taVNS markedly reduced the ACF count, with more profound effect by taVNS 2 and 3. All in all, the proposed taVNS therapy restored the colonic architecture close to normal. It would not be out of place to mention that the neoplastic lesions as observed after DMH administration were also subsided after taVNS.

Carcinogenesis is a complex phenomenon and it is settled scientific theory that loss of apoptosis is the mediator for carcinogenesis (Lowe and Lin 2000). Apoptotic pathway is majorly governed by mitochondria under the direct control of several proteins.

Categorically mitochondrial apoptotic proteins are either pro-apoptotic (BAX and BAD) or anti-apoptotic (Bcl-2 and Bcl-xl) in nature (Gross, Mcdonnell, and Korsmeyer 1999). Treatment with DMH was very well apparent for progression of colon carcinogenesis , through up-regulated expression for anti-apoptotic (Bcl-2 and Bcl-xl) signals and down-regulated expression for pro-apoptotic (BAX) signals, with more profound effect by taVNS 5.

Progression of apoptosis is further mediated through release of cytochrome c from mitochondria and formation of apoptosomes, causing increased expression for cytochrome c and decreased expression for VDAC, Apaf-1 and procaspase 9 (Estaquier J , Vallette F, Vayssiere JL 2012). Treatment with DMH down-regulated cytochrome c, with vice versa effect upon VDAC, Apaf-1 and procaspase 9 expressions. The findings from the immunoblotting assay were further affirmed through qRT-PCR studies through scrutinizing the respective phenotypes. It would not be out of place to mention that the above finding are in line with previous reports (Youle and Strasser 2008; Estaquier J , Vallette F, Vayssiere JL 2012). taVNS was very well evident to counteract the deleterious effects of DMH by inducing apoptosis, with more pronounced effect by taVNS 5.

The study was further extended to scrutinize the serum level of caspase 3 and 8 (effector caspases for execution of apoptosis). DMH treatment decreased the levels of caspase 3 and 8, which in line with previous reports (Brown et al. 2015; McIlwain, Berger, and Mak 2015). Concomitant taVNS application up-surged the levels of caspase 3 and 8. The above findings clearly endorse the induction of mitochondrial apoptosis with taVNS application.

All in all, taVNS application counteracted the carcinogenic effects of DMH, by up regulating mitochondrial apoptosis. Henceforth, it becomes imperative for us to study the effect of taVNS on CAP as per our original hypothesis.

DMH administration curtailed the α7nAchR expression at protein and m RNA level, which was up-regulated after taVNS application. The up-regulation of α7nAchR expression after taVNS application is a clear indicative for induction of CAP. The up-regulation of CAP was further substantiated by the observation that taVNS application curtailed the expression of inflammatory markers (NFκB and TNF-α). It would be appropriate to mention that taVNS 3, 4 and 5 imparted more favourable effect upon α7nAchR, NFκB and TNF-α respectively. One of the major executors for inflammatory cascade regulated by NFκB and TNF-α is HMGB1 and the same was up-regulated after the DMH treatment. It is well reported in literature that CAP curtails NFκB and subsequently downstream mediator HMGB1, which was very well evident after taVNS(Garzoni, Faure, and Frasch 2013; Brem et al. 2007). It would not be out of place to mention that taVNS 4 and 5 were equipotent to curtail the expression for HMGBI. The above findings are clear endorsement for up-regulation of CAP mediated through α7nAchR signalling as a consequence of taVNS (Figure 5).

(b). Effect of α7nAchR agonist alone and in combination with transcutaneous vagus nerve stimulation on mitochondrial apoptosis against 1,2-dimethyl hydrazine induced colon carcinogenesis in *albino wistar* rats

The connection of autonomic dysfunction with cancer is very well reported fact in previous studies. The clinical and pre-clinical studies of cancer favours the distorted ECG and lower HRV(Arab et al. 2016; Guo et al. 2013; Fadul et al. 2010). Pre clinical studies of DMH induced colon carcinogenesis confirms the above mentioned findings in reference to ECG and HRV variations in CRC (Mishra et al. 2016; Sammi

et al. 2018). It would be suitable to state that hemodynamic parameters (ECG and HRV) have turn up as non-invasive markers for autonomic dysfunction in cancer.

The treatment with PNU + taVNS was more effectively modulate the ECG parameters in compare to PNU alone. The parameters of time and frequency domain of HRV also up-regulated with PNU + taVNS treatment. All in all, PNU+ taVNS treatment was reported to regulate the autonomic dysfunction against DMH induced colon carcinogenesis. The pH, total acidity, weight variation was favourably regulated by treatment with PNU + taVNS.

taVNS III was also recorded to favourably regulate pH, total acidity; weight variation in the DMH treated animals.

Morphological characterization is an established and affirmative diagnostic technique for cancer (Shia et al. 2017). Morphological study of colon tissues DMH treated animals was reported for occurrence of ACF. Methylene blue staining together with SEM and H&E staining validate the presence of ACF and is in line with previous findings of our research (Mishra et al. 2016; Sammi et al. 2018).

The presence of small neoplastic lesion also reported in colon mucosa on observing more closely (2000 X). The treatment with PNU+ taVNS reduced the ACF count, subside neoplastic lesion with restoration of colonic morphology towards the normal control.

Various factors participate in cancer development. Oxidative stress is one of them and key regulator in development of all stages of cancer (Noda and Wakasugi 2001).Treatment with DMH results in increased levels of TBAR's and PC, GSH along with decreased levels of SOD and catalase. The TBAR's and PC are stable, reactive and sensitive marker of lipid and protein peroxidation of membrane (Tsai and Huang 2015; Shukla et al. 2014). Increased levels of TBAR's and PC in DMH treated

animals indicate the involvement of same in colon cancer development. In response to PNU + taVNS treatment, a decreased level of TBAR's and PC confirms the effectiveness in treatment against DMH induced colon carcinogenesis. SOD and catalase are the endogenous enzymes which scavenges free radicals. Superoxide radicals scavenges by SOD and produced hydrogen peroxide and molecular oxygen which subsequently catabolised in water and molecular oxygen. It is perfect to remark that SOD and catalase work in tandem as a defensive team against ROS (Kaithwas, Dubey, and Pillai 2011; Singh et al. 2008).

In present study , the decreased levels of SOD and catalase was noted with DMH treatment, could be attributed to enhanced consumption of same in response to DMH produced oxidative stress. The restoration of SOD and catalase with PNU+taVNS treatment affirms its effectiveness against DMH induced colon carcinogenesis.

Cancer can be explain as a condition in which successive genetic changes which transformed a normal cell in to malignant cell (Wong 2011). Carcinogenesis is a complicated process and loss of programmed cell death or apoptosis is the key step for carcinogenesis (Fernald and Kurokawa 2013).

Apoptosis is mainly governed by mitochondrial processes which are regulated through various protein (Lopez and Tait 2015) . Anti-apoptotic (Bcl-2 and Bcl-xl) and pro –apoptotic (BAD and BAX) are two major classes of mitochondrial proteins which involved in apoptosis. In normal condition of apoptosis , the expression of pro-apoptotic proteins found higher than anti- apoptotic proteins (Gross, Mcdonnell, and Korsmeyer 1999; Tsujimoto 1998). DMH treatment very well evident in colon cancer progression affirmed by increased expression of anti-apoptotic (Bcl-2 and Bcl-xl) and decreased expression of pro-apoptotic (BAD and BAX) markers. Treatment with PNU and PNU+taVNS, down regulate the expression of anti-apoptotic (Bcl-2

and Bcl-xl) and increase in expression of pro –apoptotic (BAD and BAX) markers , affirms its positive effect in management of DMH induced colon carcinogenesis.

In response to above mediators, cytochrome c released from mitochondria and forms apoptosomes with VDAC, Apaf-1 and procaspase 9 results in increased expression of cytochrome c and decreased expression of VDAC, Apaf-1 and procaspase 9 (Estaquier J , Vallette F, Vayssiere JL 2012). DMH treatment down regulates expression of cytochrome c along with vice-versa effect on rest of apoptosomes associated markers (VDAC, Apaf-1 and procaspase 9). Treatment with PNU and PNU + taVNS positively modulate the apoptosis against DMH induced colon carcinogenesis which was confirmed by decrease expression of anti-apoptotic proteins (Bcl-2 and Bcl-xl), VDAC, Apaf-1, procaspase 9 and up regulation of pro-apoptotic proteins (BAX and BAD) and cytochrome c.

Serum levels of caspase 3 and caspase 8 was estimated in next step of study. Caspase 3 and caspase 8 are effector caspases for execution of apoptosis. The levels of both caspase 3 and caspase 8 reduced which is in line with previous studies (Brown et al. 2015; McIlwain, Berger, and Mak 2015).Treatment with PNU+ taVNS result in restoration of caspase 3 and caspase 8 towards normal.

Treatments with DMH up regulate expression of NFκB and UCHL-1. Treatment with PNU and PNU + taVNS treatment down regulate expression of NFκB and UCHL-1. The decrease expression of inflammatory markers NFκB and UCHL-1 in response to treatment further validate the anti-inflammatory action of PNU and taVNS.

PNU is selective agonist of neural α7nAChR which binds with receptor and activates to produce the response (Hajós et al. 2005). taVNS releases Ach from nerve endings, which is well reported for anti-inflammatory activity through CAP (Zhao et al. 2012). Application of PNU with taVNS may work in synergistic manner that’s why

PNU+taVNS treatment is more effective against DMH induced colon carcinogenesis in compare to PNU.

As evident from biochemical, histopathology, imaging and various other parameters, the ^{1}H NMR based metabolomics results were concurrent. The systematic analysis revealed that the DMH control induced metabolic alteration were ameliorated upon treatments PNU and PNU+taVNS in terms of metabolic restoration alleviating the therapeutic efficacy. Thus the DMH induced metabolic changes are discussed hereafter. The decreased serum glucose to the increased lactate levels in toxicant treatment account for increased energetic and biosynthetic demand, growth and proliferation (increased glycolytic activity and lactate production, in the presence of oxygen) a phenomenon known as 'Warburg effect' consistent with cancerous cells (Mason and Rathmell 2011). As also supported by the increased levels of 3-HB (ketone bodies) and 3-Hydroxyisovalerate (3-HIVA) suggest increased ketogenesis to compensate for energy shortage under conditions of oxidative stress (Martinez-Outschoorn et al. 2012; Hao et al. 2017). Further, augmented levels of lipoproteins (VLDL), lipids, PUFAs, acetate and decreased levels of glycerol in DMH treated animals advocates the de novo glycerolipid biosynthesis important forthegeneration of biological membranes and rapid proliferatio (Zhang 2012; Beloribi-Djefaflia, Vasseur, and Guillaumond 2016; Huang and Freter 2015)**.** The reduced levels of choline might be due to accelerated demand for choline and its derivatives during rapid cell proliferation (L. Wang et al. 2013). Moreover, an aberrant choline metabolism is another major hallmark of cancer cells. Significant increases in N-acetyl glycoprotein (NAG) in rat sera indicated inflammation since NAG are well accepted as ‘‘acute phase’’ glycoproteins under inflammatory conditions (Rawat et al. 2016), as also inflammation is a most common clinical manifestation of various

cancer types. Elevated levels of allantoin is another indicator of oxidative stress and inflammation(Kanďár R , Štramová X, Drábková P 2014). Further, to meet the requirements necessary to maintain energy, cellular homeostasis, rapid cellular proliferation, and growth amino acid metabolism is specific to tumor cells. The increased serum levels of amino acids (BCAA, AAA, proline) reflect degradation of structural proteins to meet cellular needs of rapidly proliferating cancer cells (Bi and Henry 2017). The decrease in serum glutamine, glycine and formate level is consistent with other studies in cancer where it has been associated with the increased metabolic activity, which provides precursors for the biosynthesis of several biomolecules (Monteiro et al. 2016; Meiser et al. 2016). Creatine and creatinine are the key intermediate metabolites in energy metabolism; the depleted levels might be related to increased energy demand of tumor cells. The deranged metabolites represent altered cancer cell metabolism amino acid metabolism (BCAA, AAA, proline, glutamine), glycolysis or gluconeogenesis (glucose, and lactate,) and lipid metabolism (LDL, VLDL, acetate, choline, and glycerol) to compensate for energy deficit. Most of the metabolic changes in toxicant treated animals were reset back to normal after treatment PNU and PNU+ taVNS, suggesting that the treatments with PNU and PNU+taVNS has the potential to balance the metabolic abnormalities in rapidly growing cells (Figure 6).

CHAPTER 5

SUMMARY

&

CONCLUSION

The present study was undertaken to study the effect of taVNS, α7nAChR agonist (PNU) alone and in combination with taVNS against DMH induced CRC and role of CAP in same. Chemotherapy and surgery are the available treatment strategies for the management of CRC. Chemotherapy is non- specific method which affects non-cancerous tissues and results in lethal side effects. Surgical treatment is invasive and painful. Therefore, non-drug and non-surgical therapy for management of CRC is need of the day.

Previous studies also claimed effectiveness of vagus nerve stimulation in regulation of inflammation associated complications such as LPS induced inflammation and colitis (Zhao et al. 2012; Sun et al. 2013). Recently taVNS also proved its effectiveness in management depression which is a non-invasive method (Rong et al. 2012).

In the present study *albino wistar* rats were used as experimental animal. CRC was induced by administration of DMH at the dose of 30 mg / kg, weekly for 6 weeks. taVNS with variable stimulation parameters and PNU were used for treatment. After completion of scheduled treatments, animals were subjected for hemodynamic changes (ECG and HRV). Next day of ECG recordings, animals were subjected for blood collection through retro-orbital plexuses and sacrificed using light ether anaesthesia. Physiological (weight variation, pH, total acidity and ACF), biochemical (TBAR's, GSH, catalase and PC), morphological (methylene blue staining, H & E staining and SEM), western blotting, qRT-PCR and metabolomics studies were performed to affirmed the effectiveness of treatments against DMH induced colon carcinogenesis.

As per results of our study, DMH administration was very well apparent for autonomic dysfunction as observed through distorted hemodynamic profile (ECG and

HRV). taVNS as well as PNU were recorded to have significant effect upon the ECG. Mono and combination therapy also up- regulated the time and frequency domain parameters for HRV. It would be appropriate to remark that decrease in HRV has emerged as a non-invasive marker for autonomic dysfunction in cancer sufferers

Morphological characterization is one of the affirmative methods for cancer diagnosis. When considered morphologically, the colonic mucosa of the DMH treated animals were observed for the presence of ACF. The presence of ACF was confirmed by using methylene blue staining, SEM and H&E staining, and found inline with previous literature. When observed through higher magnification, small neoplastic lesions occurred in the colonic tissue. However, taVNS markedly diminished the ACF count. All in all, the proposed taVNS therapy restored the colonic architecture towards the normal. It would not be out of place to mention that the neoplastic lesions as observed after DMH administration were also subsided after taVNS.

DMH administration was also recorded for per-oxidative damage, supporting the involvement of ROS in colon cancer development. Monotherapy as well as combination therapy of taVNS and PNU resulted in significant reduction of lipid and protein peroxidation as confirmed by decrease in TBAR's and PC.

In present study, the levels of SOD and catalase were decreased in response to DMH treatment. This decrease in enzymatic activity of SOD and catalase could be attributed to increased utilization in response to oxidative stress. taVNS resulted in restoration of the SOD and catalase, confirming the say of taVNS in colon carcinogenesis.

DMH application conspicuously inhibited the mitochondrial apoptosis which was restored back after taVNS and PNU application, when scrutinized through immunoblotting and qRT-PCR studies. taVNS application up-regulated the CAP as

perceived through increased expression for α7nAchR and decreased expression for NFκB, TNF-α and HMGB-1 at protein and mRNA levels. Effect of taVNS in combination with PNU also studied through metabolomics studies.Metabolomics studies affirmed the effectiveness of combination in positive regulation of metabolic profiling against DMH induced CRC.

All in all, taVNS up-surged the CAP to counteract DMH induced colon carcinogenesis. Among all the stimulation parameters used, taVNS 3 (pulse width-1 ms, frequency-6Hz, voltage-6v, duration- 240 min.) was observed to be most effective and these stimulating parameters were used to studied the effect of taVNS in combination with PNU.

Considering anti-inflammatory, non-invasive properties of taVNS and receptor specificity of PNU, the present work hold as upper edge over currently available invasive, painful and non-specific therapeutic regimen for clinical management of CRC. Considering the effectiveness of taVNS alone as well as in combination with PNU, it could be potential therapeutic alternative for clinical management of CRC.

Moreover, the use of taVNS alone and in combination with PNU as a complimentary therapy for colon carcinogenesis is under investigation at our laboratory. Monotherapy as well combination therapy of PNU and taVNS could be further explored as strategy in the management of colon carcinogenesis; however the same needs to be validated through clinical studies.

Authors would like to conclude that taVNS alone as well as in combination with PNU imparted a significant effect upon DMH induced colon carcinogenesis mediated through CAP.

REFERENCES

Altschuler SM , Escardo J, Lynn RB, Miselis RR. 1993. "The Central Organization of the Vagus Nerve Innervating the Colon of the Rat ." *Gastroenterology* 104 (2):502–9.

Arab, Claudia, Daniel Penteado Martins Dias, Renata Thaís de Almeida Barbosa, Tatiana Dias de Carvalho, Vitor Engrácia Valenti, Tânia Brusque Crocetta, Marcelo Ferreira, Luiz Carlos de Abreu, and Celso Ferreira. 2016. "Heart Rate Variability Measure in Breast Cancer Patients and Survivors: A Systematic Review." *Psychoneuroendocrinology* 68:57–68. https://doi.org/10.1016/j.psyneuen.2016.02.018.

Balkwill F, Charles KA, Mantovani A. 2005. "Smoldering and Polarized Inflammation in the Initiation and Promotion of Malignant Disease ." *Cancer Cell* 7 (3):211–17. https://doi.org/10.1016/j.ccr.2005.02.013.

Bellinger DL , Millar BA, Perez S, Carter J, Wood C, ThyagaRajan S, Molinaro C, Lubahn sC, Lorton D. 2008. "Sympathetic Modulation of Immunity : Relevance to Disease ." *Cell Immunol. 2008* 252 (1–2):27–56. https://doi.org/10.1016/j.cellimm.2007.09.005.

Beloribi-Djefaflia, S, S Vasseur, and F Guillaumond. 2016. "Lipid Metabolic Reprogramming in Cancer Cells." *Oncogenesis* 5 (1):e189. https://doi.org/10.1038/oncsis.2015.49.

Berthoud HR , Carlson NR, Powley TL. 1991. "Topography of Efferent Vagal Innervation of the Rat Gastrointestinal." *Am J Physiol.* 260 (1 pt 2):R200-7. https://doi.org/10.1152/ajpregu.1991.260.1.R200.

Bi, X, and C J Henry. 2017. "Plasma-Free Amino Acid Profiles Are Predictors of Cancer and Diabetes Development." *Nutrition & Diabetes* 7 (3):e249. https://doi.org/10.1038/nutd.2016.55.

Bird, Ranjana P. 1987. “Observation and Quantification of Aberrant Crypts in the Murine Colon Treated with a Colon Carcinogen: Preliminary Findings.” *Cancer Letters* 37 (2):147–51. https://doi.org/10.1016/0304-3835(87)90157-1.

Bonaz, B, V Sinniger, and S Pellissier. 2016. “Anti-Inflammatory Properties of the Vagus Nerve : Potential Therapeutic Implications of Vagus Nerve Stimulation .” *J Physiol. 2016* 594 (20):5781–90. https://doi.org/10.1113/JP271539.

Borrello, Maria Grazia, Luisella Alberti, Andrew Fischer, Debora Degl’innocenti, Cristina Ferrario, Manuela Gariboldi, Federica Marchesi, et al. 2005. “Induction of a Proinflammatory Program in Normal Human Thyrocytes by the RET/PTC1 Oncogene.” *Proceedings of the National Academy of Sciences* 102 (41):14825–30. https://doi.org/10.1073/pnas.0503039102.

Brem, Harold, Olivera Stojadinovic, Robert F Diegelmann, Hyacinth Entero, Brian Lee, Irena Pastar, Michael Golinko, Harvey Rosenberg, and Marjana Tomic-Canic. 2007. “Cholinergic Anti-Inflammatory Pathway Activity and High High Mobility Group Box-1 (HMGB1) Serum Levels in Patients with Rheumatoid Arthritis.” *Molecular Medicine* 13 (9):30–39. https://doi.org/10.2119/2006.

Brown, M. F., B. J. Leibowitz, D. Chen, K. He, F. Zou, R. W. Sobol, D. Beer-Stolz, L. Zhang, and J. Yu. 2015. “Loss of Caspase-3 Sensitizes Colon Cancer Cells to Genotoxic Stress via RIP1-Dependent Necrosis.” *Cell Death and Disease* 6 (4):1–10. https://doi.org/10.1038/cddis.2015.104.

Couck M, Gidron Y. De. 2013. “Norms of Vagal Nerve Activity , Indexed by Heart Rate Variability , in Cancer Patients .” *Cancer Epidemiol.* 37 (5):737–41. https://doi.org/10.1016/j.canep.2013.04.016.

Coussens LM, Werb Z. 2002. “Inflammation and Cancer.” *Nature* 420 (6917):860–67. https://doi.org/10.1038/nature01322.

Cui, Qiu, Ian A. Lewis, Adrian D. Hegeman, Mark E. Anderson, Jing Li, Christopher F. Schulte, William M. Westler, Hamid R. Eghbalnia, Michael R. Sussman, and John L. Markley. 2008. "Metabolite Identification via the Madison Metabolomics Consortium Database [3]." *Nature Biotechnology* 26 (2):162–64. https://doi.org/10.1038/nbt0208-162.

DF, Cechetto. 1987. "Central Representation of Visceral Function." *Federation Proceedings* 46 (1):17–23.

Ee, Benarroch. 1993. "The Central Autonomic Network : Functional Organization , Dysfunction , and Perspective ." *Mayo Clin Proc.* 68 (10):988–1001.

Estaquier J , Vallette F, Vayssiere JL, Mignotte B. 2012. "Advances in Mitochondrial Medicine" 942 (Estaquier J, Vallette F, Vayssiere JL, Mignotte B.):157–83. https://doi.org/10.1007/978-94-007-2869-1_7.

Fadul, Nada, Florian Strasser, J. Lynn Palmer, Syed Wamique Yusuf, Ying Guo, Zhijun Li, Julio Allo, and Eduardo Bruera. 2010. "The Association Between Autonomic Dysfunction and Survival in Male Patients with Advanced Cancer: A Preliminary Report." *Journal of Pain and Symptom Management* 39 (2). Elsevier Inc:283–90. https://doi.org/10.1016/j.jpainsymman.2009.06.014.

Ferlay J , Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. 2015. "Cancer Incidence and Mortality Worldwide : Sources , Methods and Major Patterns in GLOBOCAN 2012 ." *Int J Cancer.* 136 (5):E359-86. https://doi.org/10.1002/ijc.29210. Epub 2014 Oct 9.

Fernald, Kaleigh, and Manabu Kurokawa. 2013. "Evading Apoptosis in Cancer." *Trends in Cell Biology* 23 (12):620–33. https://doi.org/10.1016/j.tcb.2013.07.006.

Foley, James O, and Franklin S Dubois. 1937. "Quantitative Studies of the Vagus Nerve in the Cat. I. The Ratio of Sensory to Motor ?bers." *Journal of Comparative*

Neurology 67 (1).

Garzoni, L., C. Faure, and M.G. Frasch. 2013. "Fetal Cholinergic Anti-Inflammatory Pathway and Necrotizing Enterocolitis: The Brain-Gut Connection Begins in Utero." *Frontiers in Integrative Neuroscience* 7 (August):1–9. https://doi.org/10.3389/fnint.2013.00057.

Gautam, Raju, Manjari Singh, Swetlana Gautam, Jitendra Kumar Rawat, Shubhini A. Saraf, and Gaurav Kaithwas. 2016. "Rutin Attenuates Intestinal Toxicity Induced by Methotrexate Linked with Anti-Oxidative and Anti-Inflammatory Effects." *BMC Complementary and Alternative Medicine* 16 (1). BMC Complementary and Alternative Medicine:1–6. https://doi.org/10.1186/s12906-016-1069-1.

Giri, Arvind Kumar, Jitendra Kumar Rawat, Manjari Singh, Swetlana Gautam, and Gaurav Kaithwas. 2015. "Effect of Lycopene against Gastroesophageal Reflux Disease in Experimental Animals." *BMC Complementary and Alternative Medicine* 15 (1). ??? https://doi.org/10.1186/s12906-015-0631-6.

Goehler, Lisa E, Monika Fleshner, and Linda R Watkins. 2006. "The Role of the Vagus Nerve in Cytokine - to - Brain Communication." *Annals of the New York Academy of Sciences* 840 (1):1998–99.

Goehler LE , Gaykema RP, Nguyen KT, Lee JE, Tilders FJ, Maier SF, Watkins LR. 1999. "Interleukin-1beta in Immune Cells of the Abdominal Vagus Nerve : A Link between the Immune and Nervous Systems ?" *J Neurosci.* 19 (7):2799–806. Interleukin–1beta.

Gross, Atan, James M Mcdonnell, and Stanley J Korsmeyer. 1999. "BCL-2 Family Members and the Mitochondria in Apoptosis BCL-2 Family Members and the Mitochondria in Apoptosis." *Genes & Development* 13:1899–1911. https://doi.org/10.1101/gad.13.15.1899.

Groves, Duncan A., and Verity J. Brown. 2005. "Vagal Nerve Stimulation: A Review of Its Applications and Potential Mechanisms That Mediate Its Clinical Effects." *Neuroscience and Biobehavioral Reviews* 29 (3):493–500. https://doi.org/10.1016/j.neubiorev.2005.01.004.

Guo, Y., J.L. Palmer, F. Strasser, S.W. Yusuf, and E. Bruera. 2013. "Heart Rate Variability as a Measure of Autonomic Dysfunction in Men with Advanced Cancer." *European Journal of Cancer Care* 22 (5):612–16. https://doi.org/10.1111/ecc.12066.

Gupta, Sukesh K., Swetlana Gautam, Jitendra K. Rawat, Manjari Singh, Shubhini a. Saraf, and Gaurav Kaithwas. 2014. "Efficacy of Variable Dosage of Aspirin in Combating Methotrexate-Induced Intestinal Toxicity." *RSC Adv.* 5 (13). Royal Society of Chemistry:9354–60. https://doi.org/10.1039/C4RA13640C.

Hajos, M. 2004. "The Selective 7 Nicotinic Acetylcholine Receptor Agonist PNU-282987 [N-[(3R)-1-Azabicyclo[2.2.2]oct-3-Yl]-4-Chlorobenzamide Hydrochloride] Enhances GABAergic Synaptic Activity in Brain Slices and Restores Auditory Gating Deficits in Anesthetized Rats." *Journal of Pharmacology and Experimental Therapeutics* 312 (3):1213–22. https://doi.org/10.1124/jpet.104.076968.

Hajós, M, R S Hurst, W E Hoffmann, M Krause, T M Wall, N R Higdon, and V E Groppi. 2005. "The Selective Alpha 7 Nicotinic Acetylcholine Receptor Agonist PNU-282987 Enhances GABAergic Synaptic Activity in Brain Slices and Restores Auditory Gating Deficits in Anesthetized Rats." *The Journal of Pharmacology and Experimental Therapeutics* 312 (3):1213–22. https://doi.org/10.1124/jpet.104.076968.CHRNA7.

Hanahan, Douglas, and Robert A Weinberg. 2000. "The Hallmarks of Cancer." *Cell* 100:57–70.

Hao, Juan, Tao Yang, Yang Zhou, Guo-Yuan Gao, Feng Xing, Yuan Peng, Yan-Yan

Tao, and Cheng-Hai Liu. 2017. "Serum Metabolomics Analysis Reveals a Distinct Metabolic Profile of Patients with Primary Biliary Cholangitis." *Scientific Reports* 7 (1):784. https://doi.org/10.1038/s41598-017-00944-9.

Helke CJ, Goldman W, Jacobowitz DM. 1980. "Demonstration of Substance P in Aortic Nerve Afferent Fibers by Combined Use of Fluorescent Retrograde Neuronal Labeling and." *Peptides* 1 (4):359–64.

Hr, Berthoud, and Neuhuber Wl. 2000. "Functional and Chemical Anatomy of the Afferent Vagal System ." *Auton Neurosci.* 85 (1–3):1–17. https://doi.org/10.1016/S1566-0702(00)00215-0.

Huang, Chunfa, and Carl Freter. 2015. "Lipid Metabolism, Apoptosis and Cancer Therapy." *International Journal of Molecular Sciences* 16 (1):924–49. https://doi.org/10.3390/ijms16010924.

Ja, Clancy, Mary Da, Witte Kk, Greenwood Jp, Deuchars Sa, and J Deuchars. 2014. "Non-Invasive Vagus Nerve Stimulation in Healthy Humans Reduces Sympathetic Nerve Activity ." *Brain Stimul. 2014* 7 (6):871–77. https://doi.org/10.1016/j.brs.2014.07.031.

Ja, Ricardo, and Koh Et. 1978. "Anatomical Evidence of Direct Projections from the Nucleus of the Solitary Tract to the Hypothalamus , Amygdala , and Other Forebrain Structures in the Rat ." *Brain Res.* 153 (1):1–26.

Kaithwas, Gaurav, Kiran Dubey, and K. K. Pillai. 2011. "Effect of Aloe Vera (Aloe Barbadensis Miller) Gel on Doxorubicin-Induced Myocardial Oxidative Stress and Calcium Overload in Albino Rats." *Indian Journal of Experimental Biology* 49 (4):260–68.

Kanďár R , Štramová X, Drábková P, Křenková J. 2014. "A Monitoring of Allantoin , Uric Acid , and Malondialdehyde Levels in Plasma and Erythrocytes after Ten

Minutes of Running Activity .” *Physiol Res* 63 (6):753–62.

Kim, Hong Sug, Jong Woo Lee, Young Hwa Soung, Won Sang Park, Su Young Kim, Jong Heun Lee, Jik Young Park, et al. 2003. “Inactivating Mutations of Caspase-8 Gene in Colorectal Carcinomas.” *Gastroenterology* 125 (3):708–15. https://doi.org/10.1016/S0016-5085(03)01059-X.

KJ, Tracey. 2008. “Reflex Control of Immunity .” 9 (6):418–28. https://doi.org/10.1038/nri2566.

Kloter, Evelyne, Katja Barrueto, Sabine D Klein, Felix Scholkmann, and Ursula Wolf. 2018. “Heart Rate Variability as a Prognostic Factor for Cancer Survival – A Systematic Review.” *Frontiers in Physiology* 9 (May):623. https://doi.org/10.3389/fphys.2018.00623.

Kong, Shan Shan, Jin Jun Liu, Tyzh Chang Hwang, Xiao Jiang Yu, Mei Zhao, Ming Zhao, Bing Xiang Yuan, et al. 2012. “Optimizing the Parameters of Vagus Nerve Stimulation by Uniform Design in Rats with Acute Myocardial Infarction.” *PLoS ONE* 7 (11):1–10. https://doi.org/10.1371/journal.pone.0042799.

Krumsiek, Jan, and Fabian J Theis. 2013. “Statistical Methods for the Analysis of High-Throughput Metabolomics Data Abstract : Metabolomics Is a Relatively New High-Throughput Technology That Aims at Measuring All Endogenous Metabolites within a Biological Sample in an Unbiased Fashion . The Resu,” no. January.

Kuipers, Ernst J., William M. Grady, David Lieberman, Thomas Seufferlein, Joseph J. Sung, Petra G. Boelens, Cornelis J.H. Van De Velde, and Toshiaki Watanabe. 2015. “Colorectal Cancer.” *Nature Reviews Disease Primers* 1. https://doi.org/10.1038/nrdp.2015.65.

Kumar, A., S. Gautam, J. K. Rawat, M. Singh, S. A. Saraf, and G. Kaithwas. 2016. “Effect of Palonosetron (5HT-3 Antagonist) and Pantoprazole (Proton Pump

Inhibitor) against Surgical Esophagitis Induced by Forestomach and Pylorus Ligation in Albino Rats." *Human and Experimental Toxicology* 35 (1):41–50. https://doi.org/10.1177/0960327115575759.

Kumar, Sanjit, Manjari Singh, Jitendra K. Rawat, Swetlana Gautam, Shubhini A. Saraf, and Gaurav Kaithwas. 2014. "Effect of Rutin against Gastric Esophageal Reflux in Experimental Animals." *Toxicology Mechanisms and Methods* 24 (9):666–71. https://doi.org/10.3109/15376516.2014.961215.

L, Steinman. 2004. "Elaborate Interactions between the Immune and Nervous Systems ." *Nat Immunol.* 5 (6):575–81. https://doi.org/10.1038/ni1078.

Lao, Victoria Valinluck, and William M. Grady. 2011. "Epigenetics and Colorectal Cancer." *Nature Reviews Gastroenterology and Hepatology* 8 (12):686–700. https://doi.org/10.1038/nrgastro.2011.173.

Li, Shaoyuan, Xu Zhai, Peijing Rong, Michael F. McCabe, Jingjun Zhao, Hui Ben, Xing Wang, and Shuxing Wang. 2014. "Transcutaneous Auricular Vagus Nerve Stimulation Triggers Melatonin Secretion and Is Antidepressive in Zucker Diabetic Fatty Rats." *PLoS ONE* 9 (10):1–6. https://doi.org/10.1371/journal.pone.0111100.

Li F , Chen Z, Pan Q, Fu S, Lin F, Ren H, Han H, Billiar TR, Sun F, Li Q. 2013. "The Protective Effect of PNU-282987 , a Selective α7 Nicotinic Acetylcholine Receptor Agonist , on the Hepatic Ischemia- Reperfusion Injury Is Associated with the Inhibition of High-Mobility Group Box 1 Protein Expression and Nuclear Factor κB Activation." *Shock* 39 (2):197–203. https://doi.org/10.1097/SHK.0b013e31827aa1f6.

Liu, Run-hua, Masanari Mizuta, and Shigeru Matsukura. 2004. "The Expression and Functional Role of Nicotinic Acetylcholine Receptors in Rat Adipocytes." *THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS* 310 (1):52–58. https://doi.org/10.1124/jpet.103.065037.mals.

Lopez, J., and S. W.G. Tait. 2015. "Mitochondrial Apoptosis: Killing Cancer Using the Enemy within." *British Journal of Cancer* 112 (November 2014):957–62. https://doi.org/10.1038/bjc.2015.85.

Lowe, S W, and a W Lin. 2000. "Apoptosis in Cancer." *Carcinogenesis* 21 (3):485–95. https://doi.org/10.1093/carcin/21.3.485.

Lv, Borovikova, S Ivanova, M Zhang, H Yang, Botchkina Gi, Watkins Lr, H Wang, N Abumrad, and Eaton Jw. 2000. "Vagus Nerve Stimulation Attenuates the Systemic Inflammatory Response to Endotoxin ." *Nature* 405 (6785):458–62. https://doi.org/10.1038/35013070.

Mantovani A., Balkwill F. 2001. "Inflammation and Cancer: Back to." *Lancet* 357:539–45. https://doi.org/10.1016/S0140-6736(00)04046-0.

Martinez-Outschoorn, Ubaldo E., Zhao Lin, Diana Whitaker-Menezes, Anthony Howell, Michael P. Lisanti, and Federica Sotgia. 2012. "Ketone Bodies and Two-Compartment Tumor Metabolism: Stromal Ketone Production Fuels Mitochondrial Biogenesis in Epithelial Cancer Cells." *Cell Cycle* 11 (21):3956–63. https://doi.org/10.4161/cc.22136.

Mason, Emily F., and Jeffrey C. Rathmell. 2011. "Cell Metabolism: An Essential Link between Cell Growth and Apoptosis." *Biochimica et Biophysica Acta - Molecular Cell Research* 1813 (4):645–54. https://doi.org/10.1016/j.bbamcr.2010.08.011.

Matteoli, Gianluca, and Guy E Boeckxstaens. 2013. "The Vagal Innervation of the Gut and Immune Homeostasis," 1214–22. https://doi.org/10.1136/gutjnl-2012-302550.

McIlwain, David R., Thorsten Berger, and Tak W. Mak. 2015. "Caspase Functions in Cell Death and Disease: Figure 1." *Cold Spring Harbor Perspectives in Biology* 7

(4):a026716. https://doi.org/10.1101/cshperspect.a026716.

Meiser, Johannes, Sergey Tumanov, Oliver Maddocks, Christiaan Fred Labuschagne, and Dimitris Athineos. 2016. “Serine One-Carbon Catabolism with Formate Overflow.” *Sci Adv.* 2 (10):e1601273. https://doi.org/10.1126/sciadv.1601273.

Mishra, Rakesh K., Shreesh Raj Sammi, Jitendra K. Rawat, Subhadeep Roy, Manjari Singh, Swetlana Gautam, Rajnish K. Yadav, et al. 2016. “Palonosetron Attenuates 1,2-Dimethyl Hydrazine Induced Preneoplastic Colon Damage through Downregulating Acetylcholinesterase Expression and up-Regulating Synaptic Acetylcholine Concentration.” *RSC Adv.* 6 (46). Royal Society of Chemistry:40527–38. https://doi.org/10.1039/C6RA04614B.

MKarin. 2006. “Nuclear Factor-kappaB in Cancer Development and Progression .” *Nature* 441 (7092431–6.):431–36. https://doi.org/10.1038/nature04870.

Monteiro, Márcia S., António S. Barros, Joana Pinto, Márcia Carvalho, Ana S. Pires-Luís, Rui Henrique, Carmen Jerónimo, Maria De Lourdes Bastos, Ana M. Gil, and Paula Guedes De Pinho. 2016. “Nuclear Magnetic Resonance Metabolomics Reveals an Excretory Metabolic Signature of Renal Cell Carcinoma.” *Scientific Reports* 6 (November):1–14. https://doi.org/10.1038/srep37275.

Ms, George, Sackeim Ha, Rush Aj, Marangell Lb, Z Nahas, Husain Mm, S Lisanby, T Burt, and J Goldman. 2000. “Vagus Nerve Stimulation : A New Tool for Brain Research and Therapy .” *Biol Psychiatry.* 47 (4):287–95.

Nicholas J Kassebaum, Amelia Bertozzi-Villa, Megan S Coggeshall, Katya A Shackelford. 2014. “Global , Regional , and National Levels and Causes of Maternal Mortality during 1990 – 2013 : A Systematic Analysis for the Global Burden of Disease Study 2013.” *Lancet* 384:980–1004. https://doi.org/https://doi.org/10.1016/S0140-6736(14)60696-6.

Noda, N, and H Wakasugi. 2001. "Cancer and Oxidative Stress." *Jmaj* 44 (12):535–39. https://doi.org/10.1016/0891-5849(90)90762-8.

Palma, Mariana Romanholi, Luiz Carlos, Marques Vanderlei, Fernanda Elisa Ribeiro, Alessandra Madia, Diego Giulliano, Destro Christofaro, Cristina Elena, and Prado Teles. 2016. "The Relationship between Post-Operative Time and Cardiac Autonomic Modulation in Breast Cancer Survivors." *International Journal of Cardiology* 5273 (16):360–65.

Paulon, Emma, Despoina Nastou, Francesca Jaboli, Juana Marin, Eric Liebler, and Owen Epstein. 2017. "Proof of Concept : Short-Term Non- Invasive Cervical Vagus Nerve Stimulation in Patients with Drug- Refractory Gastroparesis." *NEUROGASTROENTEROLOGY* 8:325–30. https://doi.org/10.1136/flgastro-2017-100809.

Percival Bailey, and Frédéric Bremer Published. 1938. "A SENSORY CORTICAL REPRESENTATION OF THE VAGUS NERVE : WITH A NOTE ON THE EFFECTS OF LOW BLOOD PRESSURE ON." *Journal of Neurophysiology*, 6–8. https://doi.org/10.1152/jn.1938.1.5.405.

Raj, P., M. Singh, J.K. Rawat, S. Gautam, S.A. Saraf, and G. Kaithwas. 2014. "Effect of Enteral Administration of α-Linolenic Acid and Linoleic Acid against Methotrexate Induced Intestinal Toxicity in Albino Rats." *RSC Advances* 4 (104). Royal Society of Chemistry:60397–403. https://doi.org/10.1039/c4ra10213d.

Rawat, Atul, Durgesh Dubey, Anupam Guleria, Umesh Kumar, AmitK Keshari, Swati Chaturvedi, Anand Prakash, Sudipta Saha, and Dinesh Kumar. 2016. "^{1} H NMR-Based Serum Metabolomics Reveals Erythromycin-Induced Liver Toxicity in Albino Wistar Rats." *Journal of Pharmacy And Bioallied Sciences* 8 (4):327. https://doi.org/10.4103/0975-7406.199339.

Rong, Pei-jing, Ji-liang Fang, Li-ping Wang, Hong Meng, Jun Liu, Ying-ge Ma, Hui Ben, et al. 2012. "Transcutaneous Vagus Nerve Stimulation for the Treatment of Depression : A Study Protocol for a Double Blinded Randomized Clinical Trial," 1–6.

Rosas-Ballina M, Tracey KJ. 2009. "Cholinergic Control of Inflammation ." *J Intern Med.* 265 (6):663–79. https://doi.org/10.1111/j.1365-2796.2009.02098.x.

Roy, Subhadeep, Atul Kumar Rawat, Shreesh Raj Sammi, Uma Devi, Manjari Singh, Swetlana Gautam, Rajnish Kumar Yadav, et al. 2017. "Alpha-Linolenic Acid Stabilizes HIF-1 Alpha and Downregulates FASN to Promote Mitochondrial Apoptosis for Mammary Gland Chemoprevention." *Oncotarget* 8 (41):70049–71. https://doi.org/10.18632/oncotarget.19551.

Saini, Manpreet Kaur, and Sankar Nath Sanyal. 2015. "Cell Cycle Regulation and Apoptotic Cell Death in Experimental Colon Carcinogenesis: Intervening with Cyclooxygenase-2 Inhibitors." *Nutrition and Cancer* 67 (4):620–36. https://doi.org/10.1080/01635581.2015.1015743.

Sammi, Shreesh Raj, Jitendra K. Rawat, Neetu Raghav, Ajay Kumar, Subhadeep Roy, Manjari Singh, Swetlana Gautam, et al. 2018. "Galantamine Attenuates N,N-Dimethyl Hydrazine Induced Neoplastic Colon Damage by Inhibiting Acetylcholinesterase and Bimodal Regulation of Nicotinic Cholinergic Neurotransmission." *European Journal of Pharmacology* 818 (June 2017). Elsevier B.V.:174–83. https://doi.org/10.1016/j.ejphar.2017.10.036.

Sanyal, S N, Shelly Jain, Preety Ghanghas, and Chandan Rana. 2015. "Translational Medicine Activation of Mitochondrial Apoptosis and Regulation of Ceramide Signalling by COX-2 Inhibitors in Colon Cancer" 5 (4). https://doi.org/10.4172/2161-1025.1000159.

Serafino, Annalucia, Noemi Moroni, Manuela Zonfrillo, Federica Andreola, Luana

Mercuri, Giuseppe Nicotera, Joseph Nunziata, et al. 2014. “WNT-Pathway Components as Predictive Markers Useful for Diagnosis, Prevention and Therapy in Inflammatory Bowel Disease and Sporadic Colorectal Cancer.” *Oncotarget* 5 (4):978–92. https://doi.org/10.18632/oncotarget.1571.

Shia, Jinru, Nikolaus Schultz, Deborah Kuk, Efsevia Vakiani, Sumit Middha, Neil H. Segal, Jaclyn F. Hechtman, et al. 2017. “Morphological Characterization of Colorectal Cancers in the Cancer Genome Atlas Reveals Distinct Morphology-Molecular Associations: Clinical and Biological Implications.” *Modern Pathology* 30 (4):599–609. https://doi.org/10.1038/modpathol.2016.198.

Shintaku, Tomohiro, Takayoshi Ohba, Hidetoshi Niwa, Tetsuya Kushikata, Kazuyoshi Hirota, Kyoichi Ono, Yasushi Matsuzaki, et al. 2014. “Effects of Propofol on Electrocardiogram Measures in Mice.” *Journal of Pharmacological Sciences* 126 (4):351–58. https://doi.org/10.1254/jphs.14181FP.

Shukla, Kamleshwar, Prince Raj, Arun Kumar, Mukesh Kumar, and Gaurav Kaithwas. 2014. “Effect of Monotherapy and Combination Therapy of Pantoprazole and Aprepitant in Gastric Esophageal Reflux Disease in Albino Rats.” *The Scientific World Journal* 2014. https://doi.org/10.1155/2014/183147.

Singh, Gurvinder, Anu T. Singh, Aji Abraham, Beena Bhat, Ashok Mukherjee, Ritu Verma, Shiv K. Agarwal, Shivesh Jha, Rama Mukherjee, and Anand C. Burman. 2008. “Protective Effects of Terminalia Arjuna against Doxorubicin-Induced Cardiotoxicity.” *Journal of Ethnopharmacology* 117 (1):123–29. https://doi.org/10.1016/j.jep.2008.01.022.

Skrzydlewska, E., A. Stankiewicz, M. Sulkowska, S. Sulkowski, and I. Kasacka. 2001. “Antioxidant Status and Lipid Peroxidation in Colorectal Cancer.” *Journal of Toxicology and Environmental Health - Part A* 64 (3):213–22.

https://doi.org/10.1080/15287390152543690.

Sun, Peng, Kewen Zhou, Sheng Wang, Ping Li, Sijuan Chen, Guiping Lin, Yan Zhao, and Tinghuai Wang. 2013. "Involvement of MAPK / NF-κB Signaling in the Activation of the Cholinergic Anti-Inflammatory Pathway in Experimental Colitis by Chronic Vagus Nerve Stimulation." *PLoS ONE* 8 (8):1–15. https://doi.org/10.1371/journal.pone.0069424.

T., Cooke, Kirkham N., Stainthorp DH., Inman C., Goeting N., and Taylor I. 1984. "Detection of Early Neoplastic Changes in Experimentally Induced Colorectal Cancer Using Scanning Electron Microscopy and Cell Kinetic Studies." *Gut* 25:748–55. NS -

Tanwar, Lalita, Vivek Vaish, and S N Sanyal. 2009. "Chemoprevention of 1 , 2-Dimethylhydrazine-Induced Colon Carcinogenesis by a Non-Steroidal Anti-Inflammatory Drug , Etoricoxib , in Rats : Inhibition of Nuclear Factor κ B" 10:1141–46.

Tariq, Kanwal, and Kulsoom Ghias. 2016. "Colorectal Cancer Carcinogenesis : A Review of Mechanisms Mechanisms of Carcinogenesis." *Cancer Biol Med* 13 (1). https://doi.org/10.28092/j.issn.2095-3941.2015.0103.

Tracey, K J . 2002. "The Inflammatory Reflex." *Nature* 420 (6917):853–9 ST. https://doi.org/10.1038/nature01321.

Tsai, Meng Chang, and Tiao Lai Huang. 2015. "Thiobarbituric Acid Reactive Substances (TBARS) Is a State Biomarker of Oxidative Stress in Bipolar Patients in a Manic Phase." *Journal of Affective Disorders* 173 (March 2015):22–26. https://doi.org/10.1016/j.jad.2014.10.045.

Tsujimoto, Yoshihide. 1998. "Role of Bcl-2 Family Proteins in Apoptosis: Apoptosomes or Mitochondria?" *Genes to Cells* 3 (11):697–707. https://doi.org/10.1046/j.1365-2443.1998.00223.x.

Ulrich, Eldon L., Hideo Akutsu, Jurgen F. Doreleijers, Yoko Harano, Yannis E. Ioannidis, Jundong Lin, Miron Livny, et al. 2008. "BioMagResBank." *Nucleic Acids Research* 36 (SUPPL. 1):402–8. https://doi.org/10.1093/nar/gkm957.

VICENS, Paloma, and Margarita TORRENTE and José L. DOMINGO Luis HEREDIA. 2017. "Behavioural Effects of PNU-282987 and Stress in an Animal Model of Alzheimer ' S Disease." *SYCHOGERIATRICS* 17:33–42. https://doi.org/10.1111/psyg.12189.

Wang, Hong, Man Yu, Mahendar Ochani, Carol Ann Amella, Mahira Tanovic, Seenu Susarla, Jian Hua Li, et al. 2003. "Nicotinic Acetylcholine Receptor α7 Subunit Is an Essential Regulator of Inflammation." *Nature* 421 (6921):384–88. https://doi.org/10.1038/nature01339.

Wang, L, J Chen, L Chen, P Deng, Q Bu, P Xiang, M Li, et al. 2013. "1H-NMR Based Metabonomic Profiling of Human Esophageal Cancer Tissue." *Mol Cancer* 12:25. https://doi.org/10.1186/1476-4598-12-25.

Willemze RA , Luyer MD , Buurman WA, de Jonge WJ . 2015. "Neural Reflex Pathways in Intestinal Inflammation : Hypotheses to Viable Therapy ." *Nat Rev Gastroenterol Hepatol.* 12 (6):353–62. https://doi.org/10.1038/nrgastro.2015.56.

Wishart, David S., Dan Tzur, Craig Knox, Roman Eisner, An Chi Guo, Nelson Young, Dean Cheng, et al. 2007. "HMDB: The Human Metabolome Database." *Nucleic Acids Research* 35 (SUPPL. 1):521–26. https://doi.org/10.1093/nar/gkl923.

Wong, Rebecca SY. 2011. "Apoptosis in Cancer: From Pathogenesis to Treatment." *Journal of Experimental & Clinical Cancer Research* 30 (1):87. https://doi.org/10.1186/1756-9966-30-87.

Xia, Jianguo, Nick Psychogios, Nelson Young, and David S. Wishart. 2009. "MetaboAnalyst: A Web Server for Metabolomic Data Analysis and Interpretation."

Nucleic Acids Research 37 (SUPPL. 2):1–2. https://doi.org/10.1093/nar/gkp356.

Xia, Jianguo, Igor V. Sinelnikov, Beomsoo Han, and David S. Wishart. 2015. "MetaboAnalyst 3.0-Making Metabolomics More Meaningful." *Nucleic Acids Research* 43 (W1):W251–57. https://doi.org/10.1093/nar/gkv380.

Xiong, Shiqiang, Peijian Wang, Liqun Ma, Peng Gao, Liuping Gong, Li Li, Qiang Li, et al. 2016. "Ameliorating Endothelial Mitochondrial Dysfunction Restores Coronary Function via Transient Receptor Potential Vanilloid 1-Mediated Protein Kinase A/Uncoupling Protein 2 Pathway." *Hypertension* 67 (2):451–60. https://doi.org/10.1161/HYPERTENSIONAHA.115.06223.

Youle, Richard J., and Andreas Strasser. 2008. "The BCL-2 Protein Family: Opposing Activities That Mediate Cell Death." *Nature Reviews Molecular Cell Biology* 9 (1):47–59. https://doi.org/10.1038/nrm2308.

Zhang, Feng. 2012. "Dysregulated Lipid Metabolism in Cancer." *World Journal of Biological Chemistry* 3 (8):167. https://doi.org/10.4331/wjbc.v3.i8.167.

Zhao, Yu Xue, Wei He, Xiang Hong Jing, Jun Ling Liu, Pei Jing Rong, Hui Ben, Kun Liu, and Bing Zhu. 2012a. "Transcutaneous Auricular Vagus Nerve Stimulation Protects Endotoxemic Rat from Lipopolysaccharide-Induced Inflammation." *Evidence-Based Complementary and Alternative Medicine* 2012:1–10. https://doi.org/10.1155/2012/627023.

APPENDIX-I

सैम हिग्गिनबॉटम इन्स्टीट्यूट ऑफ एग्रीकल्चर, टेक्नालॉजी एण्ड साइंसेज़
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May 10, 2016

Certificate

This is to certify that project title "Anti-inflammatory implication of vagus nerve stimulation in colon carcinogenesis" for 60 Wistar rats to Dr. Pushpraj S Gupta as Principal Investigator and Dr. Gaurav Kaithwas as Co-Principal Investigator has been approved by IAEC-SHIATS vide approval No: IAEC/SHIATS/PA16III/SJPG17.

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Allahabad, Uttar Pradesh,
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APPENDIX-II

URKUND

Urkund Analysis Result

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APPENDIX-III

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Full length article

Galantamine attenuates N,N-dimethyl hydrazine induced neoplastic colon damage by inhibiting acetylcholinesterase and bimodal regulation of nicotinic cholinergic neurotransmission

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ARTICLE INFO

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Vagus nerve

ABSTRACT

The present study reveals the effect of galantamine (GAL) against 1, 2-dimethylhydrazine (DMH) induced colon cancer. Wistar albino rats were arbitrarily divided into four groups (n = 8). Group 1 served as normal control (normal saline, 3 ml/kg/day, p.o.); group 2, 3 and 4 received DMH (20 mg/kg/week, s.c.), for 6 weeks; groups 3 and 4 also received GAL (2 and 4 mg/kg/day, p.o) for 6 weeks. DMH treated rats showed decreased heart rate variability (HRV) factors, increased incidence of aberrant crypt foci (ACF), increased thiobarbituric acid reactive substances (TBARs) along with the decrease in the enzymatic activity of superoxide dismutase (SOD) and catalase. Increased levels of inflammatory marker cyclooxygenase (COX) and lipoxygenase (LOX) was also evident in DMH treated animals. The colonic surface architecture was studied using scanning electron microscopy revealed aberrant crypts(X500) and neoplastic nodules (X2000). GAL treatment helped to minimize the ACF count, restored oxidative stress and inflammatory markers favorably. To further validate our results, our study was directed to define the effect of GAL on acetylcholine neurotransmission using a simple model organism, *Caenorhabditis elegans (C. elegans)*. Increased synaptic cholinergic transmission by GAL (32 μM) was evident in the worms when studied through aldicarb assay. However, GAL (32 μM) treatment negatively modulated α7 nicotinic acetylcholine receptor (α7nAch receptor), when evaluated using the levamisole assay. GAL (32 μM) treatment down regulated the genomic expression of *ace-1, ace-2* along with *unc-29, unc-38,* and *unc-50* (essential components of α7 nAch receptor). GAL by inhibiting AchE and regulating Alpha7nACh activity can improve cholinergic neurotransmission.

1. Introduction

The anti-inflammatory effects of the vagus nerve have been accredited to acetylcholine (Ach), which has been designated as cholinergic anti-inflammatory pathway (Jonge and Ulloa, 2007). The cholinergic anti-inflammatory pathway regulates the systemic and local inflammation through the participation of α7 nicotinic acetylcholine receptor (α7nAch receptor) on immune cells (Tracey, 2002). The α7nAch receptor is a ligand gated ion channel and regulates the production of pro-inflammatory cytokines through macrophages (Costa et al., 2012). The transcripts for α7nAch receptor have been detected in multiple anti-inflammatory cells including macrophages and the expression of α7 subunits observed to be is crucial for the anti-inflammatory effect of vagus nerve signaling (Matsunaga et al., 2001).

The anti-inflammatory potential of the cholinergic anti-inflammatory pathway has been demonstrated in the variety of preclinical studies related to sepsis, ischemia, postoperative paralytic ileus, pancreatitis and experimental colitis (Van Westerloo et al., 2005; Wang et al., 2004, 2003). Activation of the α7nAch receptor has been well documented to reduce the production of pro-inflammatory cytokines tumor necrosis factor (TNF-α), interleukin (IL-1B and IL-6). However, the production of anti-inflammatory cytokines is not affected (Nizri

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RSC Advances

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Palonosetron attenuates 1,2-dimethyl hydrazine induced preneoplastic colon damage through downregulating acetylcholinesterase expression and up-regulating synaptic acetylcholine concentration

Rakesh K. Mishra,†[a] Shreesh Raj Sammi,†[b] Jitendra K. Rawat,[a] Subhadeep Roy,[a] Manjari Singh,[a] Swetlana Gautam,[a] Rajnish K. Yadav,[a] Uma Devi,[a] Mohd Nazam Ansari,[c] Abdulaziz S. Saeedan,[c] Shubhini A. Saraf,[a] Rakesh Pandey[b] and Gaurav Kaithwas*[a]

The present study was undertaken to evaluate the effect of palonosetron (PAL) against 1,2-dimethylhydrazine (DMH)-induced colon cancer. Wistar albino rats were randomly divided into four groups ($n = 8$). Group 1 served as normal control (1 mM EDTA + saline, 2 ml per kg per day, s.c.); group 2 toxic control; group 2, 3 and 4 received DMH (20 mg per kg per week, s.c.), for 6 weeks; groups 3 and 4 also received PAL (0.25 and 0.50 mg per kg per day, p.o) for 6 weeks. DMH treated rats showed altered heart rate variability (HRV) factors, increased incidence of aberrant crypt foci (ACF), distorted antioxidant markers (TBAR's, SOD, catalase, GSH) and increased levels inflammatory markers (COX). The colonic surface architecture, studied using scanning electron microscopy (SEM), revealed aberrant crypts (500×) and preneoplastic nodules (2000×). PAL treatment helped to minimize the ACF count, and restored oxidative and inflammatory markers favorably. To further validate our results, we directed our study to define the effect of PAL on acetylcholine (Ach) neurotransmission using a simple model organism, *C. elegans*. Increased cholinergic transmission by PAL (8 μM) was evident in the worms when studied through an aldicarb assay. However, PAL (2 μM, 4 μM and 8 μM) treatment negatively modulated nAchR, when evaluated using the levamisole assay. The increased synaptic Ach levels can be attributed to the decreased levels of acetylcholinesterase (AchE), which could be attributed to the decreased genomic levels of *ace-1* and *ace-2*. The above findings were also supported by the fact, that we observed decreased AchE activity in PAL treated rats. In addition the downregulation in the expression of *unc-38* (one of the necessary components of nAchR) sufficiently links with the decreased nAchR activity. Our findings emphasize the potential role of PAL in the suppression of colon carcinogenesis.

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1. Introduction

The cholinergic anti-inflammatory pathway has been considered to be an additional endogenous mechanism to regulate the immune system and provide immediate modulatory input on the inflammatory cells. The physiological importance of the cholinergic anti-inflammatory pathway has recently immerged more strongly due to researchers indicating that stimulation of the vagus nerve could modulate systemic inflammation and the immune response.[1] A plethora of evidences are available supporting the information that activation of the vagus nerve or mimicking the cholinergic anti-inflammatory pathway can be exploited in variable disorders.[2,3] Recent studies have also demonstrated that activating the cholinergic anti-inflammatory pathway with a pharmacological agent or through vagus nerve stimulation, can confer anti-inflammatory effects both locally and systematically.[2,3] In fact, extensive research on guanyl hydrazone (CNI-1493) (centrally acting pharmacological agent) was reported to anti-inflammatory activities in experimental model of cancer, pancreatitis and arthritis.[4] Studies have also demonstrated that the peripheral anti-inflammatory effect of CNI-1493 are mediated through vagus nerve firing and subsequently modulated cholinergic anti-inflammatory pathway.

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Publications from the present research work

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2. Rakesh K. Mishra, Shreesh Raj Sammi, **Jitendra K. Rawat**, Subhadeep Roy, Manjari Singh, Swetlana Gautam, Rajnish K. Yadav, Uma Devi, Mohd Nazam Ansari, Abdulaziz S. Saeedan, Shubhini A. Saraf, Rakesh Pandey, Gaurav Kaithwas. "Palonosetron attenuates 1,2-dimethyl hydrazine induced preneoplastic colon damage through downregulating acetylcholinesterase expression and up-regulating synaptic acetylcholine concentration" *RSC Adv.,* 2016, 6, 40527–40538.

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Publications related to research work

1. Subhadeep Roy , Manjari Singh , Atul Rawat , Uma Devi , Swetlana Gautam, Rajnish Kumar Yadav, **Jitendra Kumar Rawat** , Md. Nazam Ansari, Abdulaziz S. Saeedan, Dinesh Kumar, Gaurav Kaithwas."GLA supplementation regulates PHD2 mediated hypoxia and mitochondrial apoptosis in DMBA induced mammary gland carcinoma", 96 (2018) 51–62.

2. Yadav RK, **Rawat JK**, Gautam S, Singh M, Kumar M, Ansari MN, Roy S, Saeedan AS, Kaithwas G."Antidiabetic activity of mefloquine via GLP-1 receptor modulation against STZ-NA-induced diabetes in *albino wistar* rats" *3 Biotech.* May; 8(5):240. doi: 10.1007/s13205-018-1250-y.

3. Swetlana Gautam, Atul K. Rawat, Shreesh R. Sammi, Subhadeep Roy, Manjari Singh, Uma Devi, Rajnish K. Yadav, Lakhveer Singh, **Jitendra K. Rawat**, Mohd N. Ansari, Abdulaziz S. Saeedan, Dinesh Kumar, Rakesh Pandey, Gaurav Kaithwas. "DuCLOX-2/5 Inhibition Attenuates Inflammatory Response and Induces Mitochondrial Apoptosis for Mammary Gland Chemoprevention" *Front Pharmacol*. 2018 Apr 6;9:314. doi: 10.3389/fphar.2018.00314. eCollection 2018.

4. Abdulaziz S. Al-Saeedan, Varsha Gautam, Mohd.Nazam Ansari, Manjari Singh, Rajnish K. Yadav, **Jitendra K. Rawat**, Uma Devi, Swetlana Gautam, Subhadeep Roy, Gaurav Kaithwas. 2018. "Revisiting the systemic lipopolysaccharide mediated neuroinflammation: Appraising the

effect of L-cysteine mediated hydrogen sulphide on it" *Saudi Pharmaceutical Journal*, 26,4, 520-527.

5. Gautam, Swetlana, Priyanka Singh, Manjari Singh, Subhadeep Roy, **Jitendra K. Rawat,** Rajnish K. Yadav, Uma Devi, Pushpraj S. Gupta, Shubhini A. Saraf, Gaurav Kaithwas. 2018. "Rifaximin, a Pregnane X Receptor (PXR) Activator Regulates Apoptosis in a Murine Model of Breast Cancer." *RSC Advances* 8 (7):3512–21. https://doi.org/10.1039/C7RA09689E.

6. Saeedan, Abdulaziz S., Indu Singh, Mohd Nazam Ansari, Manjari Singh, **Jitendra K. Rawat,** Uma Devi, Swetlana Gautam, Rajnish K. Yadav, Gaurav Kaithwas. 2018. "Effect of Early Natal Supplementation of Paracetamol on Attenuation of Exotoxin/endotoxin Induced Pyrexia and

7. Rani, Vidhata, Swetlana Gautam, **Jitendra K. Rawat,** Manjari Singh, Uma Devi, Rajnish K. Yadav, Subhadeep Roy, and Gaurav Kaithwas. 2018. "Effects of Minocycline and Doxycycline against Terbutaline Induced Early Postnatal Autistic Changes in Albino Rats." *Physiology and Behavior* 183 (June 2017). Elsevier:49–56. https://doi.org/10.1016/j.physbeh.2017.10.024.

8. Roy, Subhadeep, Atul Kumar Rawat, Shreesh Raj Sammi, Uma Devi, Manjari Singh, Swetlana Gautam, Rajnish Kumar Yadav, Jitendra K. Rawat et al. 2017. "Alpha-Linolenic Acid Stabilizes HIF-1 Alpha and Downregulates FASN to Promote Mitochondrial Apoptosis for Mammary Gland Chemoprevention." *Oncotarget* 8 (41):70049–71. https://doi.org/10.18632/oncotarget.19551.

9. D. Ahmed, I. Khan, G. Kaithwas, S. Roy, S. Gautam, M. Singh, U. Devi, R. Yadav, **J. Rawat**, S. Saraf. 2017. " Molecular Docking Analysis and Antidiabetic activity of Rifabutin against STZ-NA induced diabetes in *albino wistar* rats" *Beni-Suef University Journal of Basic and Applied Sciences* (2017), doi: http://dx.doi.org/10.1016/j.bjbas.2017.04.010

10. Sneha Yadav, Virendra Tiwari, Manjari Singh, Rajnish K. Yadav, Subhadeep Roy, Uma Devi, Swetlana Gautam, **Jitendra K. Rawat**, Mohd. Nazam Ansari, Abdulaziz S. Saeedan, Anand Prakash, Shubhini A. Saraf, Gaurav Kaithwas, "Comparative efficacy of alpha-linolenic acid and gamma-linolenic acid to attenuate valproic acid-induced autism-like features." *J Physiol Biochem.* DOI 10.1007/s13105-016-0532-2.

11. Asha Rani, Subhadeep Roy, Manjari Singh, Uma Devi, Swetlana Guatam , **Jitendra K. Rawat**, Mohd. Nazam Ansari, Abdulaziz S. Saeedam, Ananad Prakash, Gaurav Kaithwas. "α-chymotrypsin regulate free fatty acid and UCHL-1 to ameliorate N-methyl nitrosourease induced mammary gland carcinoma in albino wistar rats." *Inflammopharmacology*, 24 (5) , DOI 10.1007/s/10787-016-0280-5**.**

12. Virendra Tiwari, Manjari Singh, **Jitendra K. Rawat**, Uma Devi, Rajnish K Yadav, Subhadeep Roy, Swetlana Gautam, Shubhini A Saraf, Vikas Kumar, Nazam Ansari, Abdulaziz S Saeedan, Gaurav Kaithwas. "Redefining the role of peripheral LPS as a neuroinflammatory agent and evaluating the role of hydrogen sulphide through metformin intervention" *Inflammopharmacology*, DOI 10.1007/s10787-016-0274-3.

13. Chetan Manral, Subhadeep Roy, Manjari Singh, Swetlana Gautam, Rajnish K Yadav, **Jitendra K. Rawat**, Uma Devi, Md. Nazam Ansari, Abdulaziz S Saeedan, Gaurav Kaithwas. "Effect of β-sitosterol against methyl nitrosourea-induced mammary gland carcinoma in albino rats" *BMC Complementary and Alternative* (2016) 16:260 . DOI 10.1186/s12906-016-1243-5

14. Uma devi, Manjari Singh, Swetlana Gautam, **Jitendra K. Rawat**, Rajnish kumar Yadav, Shubhadeep Roy, Gaurav Kaithwas. "Experimental Models for Autistic Spectrum Disorder- Follow Up For the Validity" *Review Journal of Autism and Developmental Disorders* ; December 2016, Volume 3, Issue 4, pp 358–376

15. Raju Gautam, Manjari Singh, Swetlana Gautam, **Jitendra K. Rawat**, Shubhini A Saraf,Gaurav Kaithwas." Rutin attenuates intestinal toxicity induced by Methotrexate linked with anti-oxidative and anti-inflammatory effects" *BMC Complementary and Alternative Medicine*, 2016, 16:99.

16. Neha Sharma, Swetlana Gautam, Uma Devi, Manjari Singh, **Jitendra K. Rawat**, Nikunj Sethi, Shubhini A. Saraf , Gaurav Kaithwas."Preclinical appraisal of terbutaline analogues in precipitation of autism spectrum disorder." *RSC Adv*., 2015, 5, 39003.

17. Arvind Kumar Giri, **Jitendra K. Rawat**, Manjari Singh, Swetlana Gautam and Gaurav Kaithwas."Effect of lycopene against gastroesophageal reflux disease in experimental animals" *Complimentry and Alternatine Medicine*,2015, DOI 10.1186/s12906-015-0631-6.

18. Arun Kumar, Manjari Singh, Swetlana Gautum, **Jitendra K. Rawat**, Shubhini A Saraf, Gaurav Kaithwas. Effect of palonosetron (5HT-3 antagonist) and pantoprazole (PPI) against surgical esophagitis induced by forestomach and pylorus ligation in albino rats. *Human and Experimental Toxicology*, 2015, 1-10.

19. Sukesh K Gupta, Swetlana Gautam, Manjari Singh, **Jitendra K. Rawat,** Shubhini A Saraf , Gaurav Kaithwas," Efficacy of Variable Dose of Aspirin to Combat Methotrexate Induced Intestinal Toxicity in Albino Rats". *RSC Advances*. 2015,**5**, 9354-9360.

20. Prince Raj, Manjari Singh, **Jitendra K. Rawat**, Swetlana Gautam, Shubhini A. Saraf , Gaurav Kaithwas, Effect of enteral administration of α-linolenic acid and linoleic acid against methotrexate induced intestinal toxicity in albino rats." *RSC Advances 2014*,**4**, 60397-60403.

21. Sanjit Kumar, Manjari Singh, **Jitendra K. Rawat**, Swetlana Gautam , Shubhini A.Saraf , Gaurav Kaithwas . "Effect of rutin against gastric esophageal reflux in experimental animals". *Toxicol Mech Methods.* 2014 Dec; 24(9):666-71.

Presentations /conferences attended

1. Participation in "GOLDCON 2014: Golden Jubilee Conference of Indian Hospital Pharmacist Association" held at Babasaheb Bhimrao Ambedkar University , Lucknow, March 1-2, 2014

2. Enumerating the Ant cataract Potential of "Drshti": A Polyherbal Formulation", **Jitendra Kumar Rawat**, Sandhya Suman, Tannu Chaubey, Gaurav Kaithwas at International Pharmaceutical Conference held at Babasaheb Bhimrao Ambedkar University Lucknow, February 2-3, 2015, under the theme "Nanoformulations and Translational Research: Small getting Bigger"

3. "Integrative Therapeutics: Pharmacology of Combination of Herbal and Synthetic Anti-inflammatory Agents", **Jitendra Kumar Rawat**, Shubhini A. Saraf, Ch.V. Rao at "2nd Lucknow Science Congress" held at Babasaheb Bhimrao Ambedkar University, Lucknow, March 27-28,2014.