This is a maiden report of a bioactive pyrrole derivative (TCCP), isolated from the leaves of *T. cordifolia* illustrating significant repression of tumour cell proliferation, tumour angiogenesis and inhibition of HSPs via inactivation of HSF-1. The molecular mechanism of action of TCCP has been delineated. The molecule was shown to have specific targets such as HSF-1, AKT and ERK1/2 in order to bring about its anti-tumour activity using HSP90 as a major canonical downstream player.

Pyrrole is a prominent biologically active scaffold displaying a plethora of bioactive properties. The pyrrole ring system comprising different pharmacophores has expedited pharmaceutical research with numerous bioactive compounds. Natural products are a good source of analogs containing pyrrole rings with diverse properties. Pharmaceuticals containing a pyrrole ring structures possess potent anticancer, antibacterial, antifungal, antimalarial, antipsychotic, anxiolytic and many more pharmacologically important actions [Bhardwaj et al., 2015]. More research is needed on such pyrrole based natural compounds to exploit its therapeutic potential. With reference to anticancer agents, several pyrrole based natural compounds such as roseophilin 226, prodigiosins 227 and synthetic aryl pyrroles like LB42908 have excellent anti tumor activity [Bhardwaj et al., 2015].

Highly malignant breast cancer and several human tumors over-express HSPs. High levels of HSPs is associated with tumor cell survival and also poor prognosis. Hence downregulation of HSPs, especially HSP90 and 70 would be a prudent approach for cancer therapy especially in cancers with restricted therapeutic options. Furthermore, the transcription factor HSF-1, by itself is a robust and versatile regulator of cancer. Primarily, HSF-1 up-regulates the expression of HSPs that protect the onco-proteins required for tumorigenesis and survival, from degradation, such as mutant p53 proteins. HSF-1 regulates glycolysis and lipid metabolism, regulates different signalling pathways, such as
HuR-HIF-1, Slug, PKC, NF-κB, PI3K-AKT-mTOR, VEGF and MAPK pathways that are essential for cell proliferation, antiapoptosis, tumor angiogenesis, epithelial-mesenchymal transition (EMT), migration, invasion, and metastasis. Also, HSF-1 regulates the expression of both miRNAs and IncRNAs [Jiang et al., 2015; Santagata et al., 2011a]. The significance of HSF-1 in carcinogenesis has been established by the reduced tumor formation in Hsf1-knockout mice. HSF-1 promotes the growth of human tumor cells in culture since reduced HSF1 levels significantly reduces their proliferation and survival [Mendillo et al., 2012]. The Heat shock response (HSR) elicited by HSF-1 in cancer cells involves transcription of genes that are not induced by heat shock [Mendillo et al., 2012] leading to metastasis and death in cancers of breast, colon and lung [Scott et al., 2011].

In the present study, we have used MDA-MB-231 cancer cells, reported to be a highly metastatic, TNBC cell line. The strategy of inhibition of HSPs through HSF-1 as a treatment modality in TNBC has not been reported widely. Previously, a CRISPR/Cas9 gene knockout study with HSP90α in HIF-1α overexpressing MDA-MB-231 cells has shown that over-expression of HSP90α is vital to counteract the hypoxia-triggered cell death and to cope with the hostile hypoxic tumor environment [Dong et al., 2016]. Also, targeting the heat shock response or HSF-1 using small molecules sensitizes tumor cells to HSP 90 inhibition [Schilling et al., 2015]. The experimental data with TCCP on cell proliferation studies clearly revealed that TCCP inhibits cell proliferation and facilitates inactivation and translocation of HSF-1 from nucleus to the cytosol. However, this trend was not seen in 17-AAG treated cells, a well known HSP90 inhibitor. HSF-1 is normally found to shuttle between the nucleus and the cytoplasm but when activated by stressors, it accumulates within the nucleus as was seen in vehicle treated cells. On the contrary, based on the notion derived from the immunolocalization results that HSF-1 may be influencing the expression
of HSPs, we investigated the accumulation of HSF-1 and its phosphorylation status on Serine307 residue in the cytosolic and nuclear fractions. Our data indicates that TCCP phosphorylates HSF-1 on Ser307 in analogy to earlier report [De Bock et al., 2011]. Further, accumulation of HSF-1 in the cytoplasm and reduction in the nucleus, post TCCP treatment may be explained based on a previous study which show that phosphorylation on the two conserved serine residues, Ser307 and Ser303 on HSF-1 is essential for its association with the 14-3-3ε, leading to the repression of HSP genes due to sequestration of HSF-1 into cytoplasm away from the promoters of hsp genes. Similar results have been previously reported with localization of HSF-1 after wortmanin exposure resulting in increased cytosolic localization and decreased levels of nuclear HSF-1 after 24 h incubation in K562 cells [Mustafi et al., 2010]. Additionally, the Ser307 phosphorylated HSF-1 localization was unaltered wherein, it was found to be increased in the cytosolic as well as the nuclear fractions in a dose dependent manner. The results indicate that phosphorylation of HSF-1 on Ser307 consequently down-regulates expression of the HSPs.

Also, we have verified whether HSF-1 through a cross talk with AKT and ERK mediates downregulation of HSP90 and HSP70 expressions. Our results clearly showed that TCCP is able to regulate AKT activity by dephosphorylation of AKT at Ser437 and also phosphorylation of ERK1/2. Thus, it is clear that TCCP abrogates expression and nuclear translocation of HSF-1 and down-regulates HSP expression via AKT inactivation, which is sustained further by the phosphorylation of ERK1/2, the kinase responsible for inactivation of HSF-1. Thus, TCCP could effectively inhibit HSP gene expression by concerted action of both AKT and ERK1/2 on HSF-1. Similar results of blockade of AKT phosphorylation by the PI3K inhibitor wortmanin revealed that HSP70 expression was dependent on activated AKT [Mustafi et al., 2010].
Our data on gene expression analysis using qPCR revealed that TCCP was able to decrease the transcription of HSP90 to a considerable extent compared to that of HSP70. However, TCCP treatment had very little effect on the levels of HSF-1 transcripts. On the other hand, 17-AAG drastically increased the HSP70 transcripts and did not alter either HSP90 or HSF-1 transcription. Furthermore, TCCP inhibited the migration of cells in a dose dependent manner as seen in the wound healing assay. This might be due to the fact that HSF-1 regulates the migration and EMT pathway and it is also being identified as one among the six-potent tumor metastasis promoting genes [Scott et al., 2011].

ROS is a by-product of oxygen metabolism in mitochondria and is continuously produced in all aerobic organisms. The dysfunction of electron transport chain causes excessive release of ROS from the mitochondria. The disproportionate production of antioxidants and ROS occurs due to stress in cancer cells. The results of this study apparently demonstrate the elevated ROS and intracellular calcium generation in MDA-MB-231 cells upon TCCP treatment indicating that TCCP may exert cytotoxic oxidative stress and act as an apoptosis modulator.

Apoptosis by both intrinsic and extrinsic pathways ultimately stimulate a set of cysteine-aspartic proteases called caspases. The caspases interact with inhibitors of apoptosis proteins (IAP) and the Bcl-2 family of proteins which are regulated by SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pi). Therefore, targeting cancer by apoptosis induction is currently the classic therapeutic aim of most cancer therapies [Nonnenmacher et al., 2016].

Mitochondria participate in the cellular energy metabolism and also play a critical role in the intrinsic pathway of apoptosis and are the prime targets of cellular oxidative stress, preventing electron transport chain leading to an amplification of ROS generation.
and opening of mitochondrial permeability transition pore. As mitochondria being the primary source of ROS production, they are highly susceptible to additional ROS attacks resulting in dissipation of $\Delta \Psi m$ and peroxidation of cardiolipin which is a major component of mitochondrial membrane. Studies show that ROS initiates apoptotic events in cells through the mitochondrial pathway that results in the depolarization of $\Delta \Psi m$ [Leytin, 2012]. Therefore, to assess the influence of TCCP on mitochondria, the $\Delta \Psi m$ was determined which substantially dissipated the $\Delta \Psi m$, indicative of the lethal effects of TCCP on mitochondria, thereby facilitating the ensuing events of apoptosis. An elevated level of intracellular Ca$^{2+}$ is one of the major contributing factors towards the changes in $\Delta \Psi m$. The accumulation of oxidized cardiolipin on the outer mitochondrial membrane results in recruitment of Bax and formation of the MPTP, which releases cytochrome c (Cyt c) from mitochondria [Li et al., 2015].

The critical step in the cytosol, released cytochrome c binds to Apaf-1 to form the apoptosome. The apoptosome complex then activates caspase-9, which in turn activates caspase-3 [Jiang and Wang, 2004]. Therefore, the next set of experiments targeted the influence of TCCP over caspase activity. The results of the study firmly highlight the inhibitory effect of TCCP on the activities of caspase 9 and 3. ROS may also activate nuclear transcription factors, like NF-$\kappa$B, activator protein-1 (AP-1), and p53, which may upregulate death proteins or produce inhibitors of survival proteins [Reuter et al., 2010].

TCCP induced opening of the mitochondrial permeability transition pore dramatically altering the permeability of mitochondria. Continuous pore activation resulted from mitochondrial Ca$^{2+}$ overload, increased levels of reactive oxygen species in mitochondria, and other pro-apoptotic conditions. Cytochrome c release from mitochondria and loss of mitochondrial membrane potential were observed subsequent to
continuous pore activation. TCCP treatment also resulted in Bax upregulation, Bcl-2 downregulation.

p53 is the most extensively studied and commonly mutated tumour-suppressor gene in more than half of human cancers, including breast cancer. Mutant p53 protein (tetrameric transcription factor) is thought to promote tumor cell survival and resistance to chemotherapeutic drugs. Therefore, restoring p53 function by converting existing mutant p53 to the wild-type p53 conformation is being pursued as one of the strategy to promote apoptosis of tumor cells. PRIMA-1 (p53 re-activation and induction of massive apoptosis) is a non-toxic small molecule that converts mutant p53 to the active conformation and induces apoptosis in tumor cells [Liang et al., 2009]. Previous report suggests that taxol, a natural compound induces G2/M arrest and apoptosis in human breast carcinoma cells (MCF-7 and MDA-MB-231), mediated through the ER and p53-independent upregulation of p21 [Choi and Yoo, 2012]. However, in our study, the expression of p53 in the MDA-MB-231 cells was not affected by TCCP; nonetheless, treatment with TCCP consistently increased the level of phosphorylation (Ser15) of p53 with respect to the untreated control cells. The p53 tumor suppressor is a short-lived transcription factor that is stabilized and activated in response to a range of cellular stresses including hyper-proliferation and DNA damage. Ser15 is the primary target of the DNA damage response on the p53 protein. p53 inhibition by pifithrin-µ reversed the effects of TCCP and promoted cell survival by attenuation of apoptosis.

It was found that the cell death induced by TCCP exhibited a clear morphological sign of apoptosis. After being treated with TCCP, cell shrinkage, chromatin condensation, and DNA ladder formation due to fragmentation were observed. These observations suggest that TCCP may be an effective chemotherapeutic agent against breast cancer.
Further studies were performed to elucidate the mechanism underlying the inhibition in cell viability and induction of apoptosis. It has been proposed that DNA fragmentation results from the loss of the compartmentalization of DNase I, which would reach the nucleus due to the breakdown of the endoplasmic reticulum and the nuclear membrane [Vermeulen et al., 2005]. Fragmentation of DNA at the internucleosomal linker regions has been observed in cells undergoing apoptosis induced by a variety of agents. This cleavage produces ladders of DNA fragments that are the size of integer multiples of a nucleosome length (180–200 bp) [Higuchi, 2003]. Since their characteristic patterns are shown by agarose gel electrophoresis, these nucleosomal DNA ladders are widely used as biochemical markers of apoptosis. These results have been further bolstered by the cellular staining by annexin V, an increase in the number of apoptotic cells were recognized with the increasing concentration of TCCP.

Further, from our in vivo EAT mouse model studies; it was evident that TCCP significantly suppressed HSP expression in the tumor bearing mouse as observed by immunohistochemistry and western blot analysis. TCCP also significantly inhibited EAT tumor growth and suppressed tumor angiogenesis. Inhibition of HSF-1 strongly inhibits secretion of VEGF, the major growth factor responsible for neovascularization, as well as other targets such as Hypoxia-inducible factor 1-alpha (HIF-1α), carbonic anhydrase IX (CAIX), and Glucose transporter 1 (Glut-1) [Gabai et al., 2012]. An angiogenesis regulating mechanism mediated by HSPs has been reported wherein; HSP90-HSP70 complex is involved in targeting the VEGFR2 receptor for degradation. Geldanamycin blocks VEGF-A signaling. Inhibition of HSP90 results in degradation of VEGFR2 by HSP70 by the endosome-lysosome degradation pathway [Bruns et al., 2012]. Comprehensively, the net result of TCCP action precipitates down to the inhibition of tumor induced angiogenesis in
an ascites tumor model where, we have shown that TCCP inhibits tumor cell proliferation, ascites secretion and peritoneal angiogenesis as is validated by an endothelial cell proliferation marker, CD31 staining and MVD scoring. Further, TCCP effectively inhibited VEGF induced neovascularization even in a non-tumor context. Our data on inhibition of angiogenesis in rat corneal micro-pocket assay in presence of VEGF further substantiated the antiangiogenic activity of TCCP.

An effective and acceptable chemopreventive or anticancer agent should have certain properties like nontoxic effects in normal and/or healthy cells, high efficacy against multiple cancers and low cost [Aziz et al., 2003]. Compiling data from in vitro and in vivo laboratory studies, epidemiological investigations, and human clinical trials indicate that phytochemicals like resveratrol [de la Lastra and Villegas, 2007], curcumin [Kim et al., 2016], green tea polyphenols, epigallocatechin-3-gallate [Hsu et al., 2003a, b], quercetin [Li et al., 2014] that are powerful antioxidants have important effects on cancer chemoprevention and therapy. The differential impact of these compounds on oxidative status in normal versus tumor cells may support the hypothesis of multiple signalling pathways that promotes tumor cells to undergo apoptosis but direct the normal epithelial cells toward a survival pathway associated with cell differentiation. However, the appropriate reasons behind the dual nature of phytochemicals as an antioxidant in normal cells while prooxidant in cancer cells requires further investigations.

Number of studies has documented biological activities of T. cordfolia against various diseases, but there is paucity of information, especially against DNP induced oxidative stress with reference to lipid peroxidation and other antioxidant marker’s status. As there is no specific antidote or specific management for individuals with DNP-related
toxicity, the present study was conducted to evaluate the protective effect of TCCP against the oxidative stress induced by DNP on blood components.

For many years, a large number of natural compounds of varied structures derived from medicinal plants have been suggested as the major source of antioxidants and are capable of exerting protective effects against oxidants and inflammation in biological systems. *T. cordifolia* has been traditionally used as herbal medicine for the treatment of various illnesses that involve inflammation and oxidative stress [Stanely Mainzen Prince and Menon, 2001; Patgiri et al., 2014; Tiwari et al., 2014]. It is well known that the anti-inflammatory and antioxidant activities are closely interrelated. The free radical scavenging activity of TCCP was found to increase with its increasing concentration.

Generally, in RBCs, at normal physiological conditions, the reaction of heme with peripheral environment is controlled since it resides inside the pockets of heme proteins. However, during oxidative stress by pro inflammatory chemicals like DNP, hemoglobin tends to release its heme prosthetic groups. For this reason, the free heme produced becomes highly toxic to the cells by generating free radicals due to reduction of ferric (+3 ion) to ferrous (+2) form which take part in Fenton reaction contained within its protoporphyrin IX ring. Within the RBC, deoxygenation of haemoglobin (Hb) is followed by the generation of O$_2^-$. This reaction occurs to a lesser extent in normal RBC. But during stress, much more O$_2^-$ is generated leading to denaturation, precipitation, haemachrome formation and ROS production [Nur et al., 2011; Iuchi, 2012]. It is known that iron catalyzes the formation of ROS and thus alters the cellular redox state. Another plausible cause for oxidative stress in RBC may be the high concentrations of iron and iron-containing compounds, such as heme and Hb, in the plasma owing to haemolysis and blood transfusions [Daghman et al., 1999].
Exposure of RBCs, platelets and polymorphonuclear leukocytes (PMN) to iron or heme increases their ROS level. The chronic oxidative stress of RBC, PMN and platelets in DNP treated blood renders them more susceptible to endogenous ROS mediated damage. Oxidative stress triggers eryptosis which outlines cell shrinkage, membrane blebbing and phospholipid damage of the cell membrane leading to phosphatidylserine translocation to the erythrocyte surface [Zierle et al., 2015]. Thus, the free radicals generated affects the neighbouring cellular components by peroxidation of unsaturated fatty acids in membranes, protein degradation and DNA fragmentation [Blokhina et al., 2003]. Several groups have suggested that molecules, which stimulate formation of ROS, can trigger apoptosis [Kelso et al., 2001; Han et al., 2007; Park et al., 2007]. The exact mechanisms involved in cell death induced by ROS are not fully understood, and the protective effect mediated by some antioxidants remains controversial. The results in the present study show that pretreatment with TCCP substantially reduced ROS and H$_2$O$_2$ induced by DNP compared to DNP alone treated RBCs. In fact, the aqueous extract of *T. cordifolia* was found to inhibit fenton reaction in a dose dependent manner with an IC$_{50}$ value of 700 mg/ml [Rana, 2002].

Lipid peroxidation is considered to be the key event that indicates marked oxidative stress. Lipid peroxidation refers to the oxidative degradation of lipids. MDA, together with 4-HNE, is a natural bi-product of lipid peroxidation and its quantification is generally used as marker for lipid peroxidation. One of the major endogenous antioxidant produced by the cells is GSH, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms. Data from this study clearly indicated that TCCP could inhibit lipid peroxidation and elevated GSH content in DNP treated RBCs. On the other
hand, DNP treated RBCs showed elevated lipid peroxidation and drastic depletion in GSH content. Likewise, oral administration of aqueous extract of the roots from *T. cordifolia* resulted in a significant reduction in thiobarbituric acid reactive substances (TBARS) and an increase in GSH, catalase and SOD in alloxan diabetic rats [Stanely et al., 2000].

There was an increase in level of both catalase and LDH in DNP treated serum due to erythrocyte damage. Catalase is an intracellular, peroxisomal and antioxidant enzyme that degrades hydrogen peroxide into water and oxygen. It is possible that intracellular catalase may be released outside the cells, if the cells are injured and further stimulate neighbouring cells to produce COX-2 and iNOS. The root extract of *T. cordifolia* was reported to decrease the concentration of glycosylated hemoglobin, plasma thiobarbituric acid reactive substances, GSH, SOD and catalase activity in heart and brain of diabetic rats [Prince et al., 2004; Umamaheswari and Prince, 2007].

LDH is abundant in RBCs and acts as a marker for hemolysis. Reports available on root extract of *T. cordifolia* showed that it can lower hepatic glucose-6-phosphatase and serum acid phosphatase, alkaline phosphatase, and LDH in diabetic rats [Stanely et al., 2000]. In the same way, the current results define the low level of catalase and LDH in TCCP pre-incubated DNP treated serum. The induction of the enzymatic and non-enzymatic defensive mechanism on exposure to DNP could be an adaptive or a counteractive response that enables the cells to overcome the damage. From a previous clinical report, patient with symptoms of tachypnea and hyperthermia post consumption of DNP also showed increased LDH concentration (768U/L). The increase in serum LDH is an indirect evidence of hemolysis [Lee et al., 2014].

The decreased level of tissue enzymes, i.e., SOD, catalase and increased level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase
ALP, and ACP were observed in mice treated with lead. Oral administration of aqueous stem extract and aqueous leaves extract from *T. cordifolia* along with the lead nitrate (5mg/kg body weight, i.p. for 30 days) increased the activities of SOD and CAT and decreased the levels of AST, ALT, ALP, and ACP enzymes in mice [Sharma and Pandey, 2010]. Correspondingly, our results demonstrated the protective efficacy of pyrrole derivative against DNP-induced oxidative damage by stabilizing and reducing the levels of ACP and ALP in blood components.