DISCUSSION

Medicinal value of MC has been attributed to its high antioxidant property which is related to phenols, flavonoids, isoflavones, terpenes, anthroquinones, and glucosinolates. Based on the previous references which have stated that ethanol is a better solvent for extraction of phytochemicals [169,170], we have used ethanol as a solvent for extraction to evaluate phytoconstituent activities and biological activities of MC. Amira K et al have shown that the pure solvents were inefficient extraction media for antioxidant and enhanced extraction yields were obtained from solvent containing a higher concentration of water [171]. Koffi E et al in their work have shown that since the vast majority of polyphenols are not water soluble manufacturers would have to use extraction solvents with a mixture of suitable solvents to obtain fractions rich in polyphenols [170]. Based on these studies, we have used different a percentage of ethanol i.e, 50%,70% and 100% for extraction and the result obtained in our study go in accordance with these references.

Phenolic compounds are known to have antioxidant activity and it is due to the redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [172]. Flavonoids are the largest group of naturally occurring phenolic compounds, which occur in different parts of the plant, both in free-state and as glycosides. The polyphenolic nature of flavonoids acts as an antioxidant which scavenges injurious free radicals such as superoxide and hydroxyl radicals. MC is a good source of phenolic compounds which possess potent antioxidant activity. Semiz A et al have shown that in-vivo treatment of rats with MC fruit extract enhanced both antioxidant enzyme (AOE) and Glutathione S-Transferase (GST) activities [173]. Hamissou M et al have shown that MC was 82.05% as effective as ascorbic acid in inhibiting the free radical DPPH and has higher total phenolic compounds, indicating that MC is a high antioxidant containing vegetable food compared to zucchini, in aqueous extracts [174]. Even though in these studies they have shown that MC is having phenol content and antioxidant activity, they have used either crude extract or single percentage of extract. But in our study, we have used a different percentage of extract and estimated the TPC, TFC, antioxidant activity and anti-inflammatory activity.

The TPC was estimated using F-C method using gallic acid as standard. A linear response was observed between 5 – 60 µg/µl and TPC was expressed as GAE(R²=0.9236). The result obtained in our study showed that the 50% (0.029%) EEMC is having highest phenol content followed by 70% (0.0098%) and 100% (0.0022%) EEMC. The TFC was estimated using Aluminum Chloride Colorimetric Method using quercetin as standard (R²=0.9985). The result obtained in our study showed that the 50% (22%) EEMC is having highest flavanoid content followed by 70% (14.5%) and 100% (11%) EEMC (Fig 27).

Moure, A et al showed that, as the polarity of the solvent increased, extraction yields of total soluble solids and total extractable polyphenolics were higher [175]. The data obtained in our work agrees with this study. The result of TPC and TFC shows that the reactivity of phenolic compounds depends upon the polarity of the medium, as there is a decrease in the polarity, reactivity also decreases proportionally showing minimum reactivity with 100% ethanol soluble fractions of phenolic contents. Further phenolic acid profiling using HPLC will be done in order to specify the key compound present in the 50% EEMC.

The reducing sugar was estimated by DNS method using D-Glucose as standard (R²=0.9920). The result obtained showed that the 50% (2.3%) EEMC is having the highest percentage of reducing sugar followed by 70% (0.51%) and 100% (0.06%) EEMC (Fig 28).

Since the 50% EEMC has shown the highest percentage of reducing sugar qualitative estimation of reducing sugar was done by ascending paper chromatography using glucose, fructose, xylulose and maltose as standard. The result obtained showed the presence of glucose in 50% EEMC (Fig 29 and Table13). The presence of glucose was further confirmed by the GOD-POD method using D-glucose as standard, which surprisingly showed 332mg/dl
of glucose in the extract. The GOD-POD method is a specific method for estimation of D-Glucose since the enzyme specifically oxidizes only D-Glucose.

There are several methods to determine antioxidant activity of plants. The antioxidant activity of different percentage of EEMC in our study was determined using three methods i.e, FRAP, DPPH and reducing power assay. The phytochemicals which are responsible for the scavenging activity are the phenolic and flavonoid content in the extract [176]. A higher absorbance of the reaction mixture indicates higher reductive potential [177]. The result obtained in our study showed that the 50% ethanolic extract had the highest antioxidant activity followed by the 70% and 100% ethanolic extracts respectively, in FRAP (Fig 30), DPPH (Fig 31) and Reducing Power Assay (Fig 32), which may be attributed to the presence of higher percentage of phenolic acids and flavonoids.

IC(50) value is defined as the concentration of antioxidant required for 50% scavenging of DPPH radicals, which is a parameter widely used to measure antioxidant activity. Smaller the IC (50) value corresponds to a higher antioxidant activity of the plant extract [178]. The data obtained in our study showed that the 50% (11.43µg/ml) EEMC has lowest IC (50) value than 70% (16.42 µg/ml) EEMC and 100% (175.4µg/ml) EEMC and attributes to TPC and TFC (Fig 33). The DPPH radical scavenging activity of EEMC increased gradually as the concentration increased. The decrease in absorbance of DPPH solution indicated by a change in the colour from purple to yellow depends on the intrinsic antioxidant activity of antioxidant and on the speed of reaction between DPPH and antioxidant present in the extract.

The anti-inflammatory activity of EEMC was estimated by HRBC Membrane Stabilization Assay using diclofenac sodium as a positive control. The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization by the extract implies that the extract will stabilize lysosomal membranes which will, in turn, limit the inflammatory response by preventing the release of lysosomal constituents. Che-Yi Chao et al and Shin-You Ciou et al have independently shown that MC in sepsis-induced mice reduced expression of proteins associated with inflammation like Cyclooxygenase-2 (COX-2), Inducible nitric oxide Synthase (iNOS), and Nuclear factor-kappaB (NF-ƙB) and reduced secretions of pro-inflammatory cytokines and other substances, hence reducing organ damage [179,180]. Umukoro S et al have shown that aqueous leaf extract of MC inhibits the late phase of inflammatory events, namely the release of chemical mediators and thus suggesting that it may offer some beneficial effects in the management of inflammatory conditions [181]. In these studies, they have used crude extract or single percentage of extract and explained about the anti-inflammatory activity of MC, but in our study, we have used a different percentage of extract for the anti-inflammatory activity. The data obtained in our study showed that the 50% ethanolic extract had the highest anti-inflammatory activity compared to 70% and 100% ethanolic extracts respectively (Fig 34). The extracts might have exhibited membrane stabilization effect by inhibiting hypotonicity induced lysis of erythrocyte membrane. Thus 50% EEMC is having more potent anti-inflammatory activity than 70% and 100% EEMC.

Thus, the data obtained showed that 50% EEMC is having highest antioxidant activity and anti-inflammatory activity when compared to 70% and 100% EEMC. Further studies will be done to know the key antioxidant compound in the extract by phenolic acid profiling using HPLC.

Hypoglycemic activity of fresh BMJ is due to the bioactive components, charantin (a steroid glycoside), vicine and polypeptide "p" or plant insulin (a 166 residue insulin mimetic peptide). Mechanisms of action include increased insulin-like effects, stimulation of pancreatic secretion, leading to decreased hepatic gluconeogenesis, increased hepatic glycogen synthesis and increased peripheral glucose oxidation [16].
Fresh BMJ has a good hypolipidemic effect. *Momordica charantia* increases the activity of adenosine 5 monophosphate kinase (AMPK), an enzyme that regulates cellular glucose uptake and fatty acid oxidation. They decrease liver secretion of apolipoprotein B (Apo B) – the primary lipoprotein of low-density cholesterol; also reduces apolipoprotein C-III expression, the protein found in very-low density cholesterol which turns into LDL/bad cholesterol; and increases the expression of apolipoprotein A-1 (ApoA1) - the major protein component of high density "good" cholesterol. It also decreases cellular triglyceride [182].

Different studies have tried to study the oral hypoglycemic and hypolipidemic effects of *Momordica charantia* using various parts of the plant. Studies using oral administration of aqueous extract [Bano F et al. 2011] over a period of five weeks showed significant decrease in blood glucose (17%, $P<0.01$), cholesterol (21% $P<0.01$), triglyceride (20% $P<0.01$), LDL cholesterol (20% $P<0.01$) and increase in HDL (45% $P<0.05$) [182]. Similarly, Fernandes N et al. (2007) have shown that oral administration of *Momordica charantia* extract showed a significant reduction in cholesterol, triglyceride, LDL-CH, VLDL-CH and blood glucose levels as compared to untreated diabetic rats [13]. In another study conducted by Chaturvedi et al. (2005) it has been shown that administration of methanol extract from dried fruits significantly decreased triglyceride and LDL and increase in HDL ($p<0.001$) [10].

The body weight of diabetic control rats was decreased whereas the body weight gradually increased in pioglitazone-treated rats and a slight reduction in the bitter melon treated group.

Though most of the studies have tried to study the glucose and lipid-lowering action of MC, the mechanisms by which they are attained still remains largely obscure and the focus of recent studies has been the same [183]. Since PPAR is a key modulator of adipogenesis and lipolysis there have been studies which have studied the effects of MC extract on PPAR [184]. PPARγ is present in adipocytes and is a master regulator of adipogenesis and in vivo acts as an insulin sensitizer, as evidenced by the effects of TZDs as PPARγ agonists [87]. The differentiation of pre-adipocytes into adipocytes is regulated by a complex network of transcription factors including PPARγ, and C/EBPs and β. PPARγ and C/EBPα activate the expression of genes involved in adipogenesis to trigger fatty acid and triglyceride syntheses via activation of fatty acid synthase (FAS), lipoprotein lipase (LPL), adipocyte differentiation-related protein (ADRP), adipocyte-specific binding protein 2 (aP2), CD36, perilipin, and others during the terminal stage of pre-adipocyte differentiation [185-187], whereas C/EBPβ activates genes involved in the initial stage of differentiation. The PPARγ1, PPARγ2 and SREBP1 mRNA levels in the adipose tissue were significantly high in the diabetic control rats when compared to the normal control rats. The expression of these transcriptional factors increased upon treatment with pioglitazone, however on treatment with MC the levels decreased.

Adipogenesis is a complex process in which adipocyte growth and differentiation contribute to overall adipose mass [188]. The experimentally-induced adipocyte
differentiation was characterized by plumping of adipocytes due to the accumulation of lipid droplets and a higher percentage of adipocyte differentiation.

LI85008F, a novel herbal formulation, has been shown to antagonize PPARγ through Ser112 phosphorylation via mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) activation. It has also been shown to downregulate the expression of PPARγ-responsive gene products such as adipocyte differentiation-related protein (ADRP), CD36, adipocyte-specific-binding protein (aP2), and perilipin. These observations also showed that LI85008F treatment resulted in decreases in fat storage and availability of the fatty acid transport system, and increased susceptibility to hormone-sensitive lipase (HSL) for the lipolytic breakdown of stored fat [189]. Nerurkar PV et al, showed that pre-adipocytes treated with varying concentrations of MC during differentiation demonstrated a significant reduction in lipid content and mRNA expression of adipocyte transcription factors including PPARγ, SREBP-1c, adipocytokine, and resistin [142]. Also, adipocytes treated with MC for 48 h showed reduced lipid content, perlipin mRNA expression, and increased lipolysis [17].

In our study, the qualitative analysis by Oil Red O staining of adipogenesis both during and after differentiation showed a decrease in the accumulation of lipid droplets with increased EEMC concentrations (Fig 38). The result of the quantitative analyses of both “during” (p = 0.012) and “after” (p = 0.026) adipocyte differentiation also showed that as EEMC concentration increased, adipogenesis decreased (Figs. 39a and 39b). The percentage of adipogenesis was less during adipocyte differentiation than after. The results of both the quantitative and qualitative analyses may be due to downregulation of the transcription factors C/EBP and PPAR family members, which slow the de novo synthesis of fatty acids and triglycerides and inhibit the early differentiation of pre-adipocytes and adipogenesis in mature adipocytes.

Adipolysis is the breakdown of lipids, which involves hydrolysis of triglycerides into glycerol and free fatty acids (FFAs). Hormone-sensitive lipase, a rate-limiting enzyme in adipose tissue lipolysis, hydrolyzes the stored triglycerides into glycerol and FFAs [190]. Catecholamines stimulate adipocyte lipolysis by increasing intracellular cAMP, an important second messenger in the signaling pathways that mobilize fat stores and activate cAMP-dependent protein kinase A (PKA). Protein kinase A then phosphorylates both perilipins and HSL, which is associated with an increase in hydrolytic activity of the enzyme and the translocation of HSL from the cytosol to the lipid droplet. It has been shown that berberine, a hypoglycemic agent, increases phosphodiesterase (PDE) 3B and PDE4 activity by reducing the inhibition of PDE, leading to a decrease in cAMP and HSL phosphorylation, which is independent of the adenosine monophosphate-activated protein kinase (AMPK) pathway [191]. The cytokine TNF-α, an important mediator of lipid metabolism, downregulates the antilipolytic genes PDE3B and Gαi [111].

This regulation is influenced by the glucose concentration to which the cells are exposed and one or more glycolytic intermediates [192].

The adipolysis was analyzed using glycerol as the standard (R² = 0.9963). Treating the cells during differentiation showed that as the EEMC concentration increased, glycerol release decreased. This may be due to decreased lipid accumulation in the cells which were under the influence of EEMC during differentiation. However, treating the cells with increasing concentrations of EEMC after differentiation increased the glycerol release since there was equal lipid accumulation in all the cells before they were exposed to EEMC. This result obtained may be attributed to a decrease in HSL phosphorylation or TNF-α productions. These findings provide evidence for the potential of EEMC in preventing adipocyte differentiation and lipid accumulation and promoting lipolysis.
Hence we conclude that 50% EEMC reduced lipid droplet accumulation and increased glycerol release on treating 3T3-L1 pre-adipocyte cells both during and after differentiation. However, the effect of EEMC on adipogenesis was greater during differentiation than after.

Akt is a key enzyme in the insulin signaling pathway in adipocytes, and the insulin-stimulated phosphorylation of Akt plays critical roles in insulin-induced glucose metabolism, glucose transport and adipocyte differentiation [193,194]. The overexpression of Akt results in increased glucose uptake and adipocyte differentiation and the inhibition of Akt expression blocked adipocyte differentiation in 3T3-L1 preadipocytes [195]. Preadipocyte differentiation is controlled by a subtle balance of serial and interdependent transcription factors [196]. C/EBPδ and C/EBPβ are rapidly and transiently expressed during the early stages of adipocyte differentiation, prior to the transcriptional activation of adipocyte-specific genes. These genes act synergistically to induce the expression of C/EBPα and PPARγ, which promotes the expression of a set of genes involved in adipocyte maturation and differentiation [197]. Akt appears to participate in the insulin signaling pathway through the phosphorylation of GSK3β and by stimulating GLUT4 translocation [198].

The Akt signaling cascade is considered important for adipogenesis, as it appears to activate PPARγ and C/EBPα during the induction of 3T3-L1 adipocyte differentiation [199]. Xu et al. showed that the expression of Akt induces an important association between the PI3-kinase-PKB/Akt signal cascade and the transcription factors PPARγ and C/EBPα in the induction of 3T3-L1 adipocyte differentiation [194]. Taken together, our results revealed that the suppression of adipogenesis by MC was caused by the decreased levels of SREBP-1 through GSK-3β, and also decrease in the expression of PPARγ. This was further confirmed on studying the mRNA expression of PPARγ and SREBP1 which also showed decreased levels on treatment with MC. Our results demonstrated that MC strongly suppressed the expression of critical genes involved in creating and maintaining the adipocyte phenotype and reduced lipid storage and accumulation in 3T3-L1 preadipocytes.

The tuberous sclerosis complex 2 (TCS2) is a critical downstream substrate of Akt in the induction of adipocyte differentiation. A critical role for Akt2-TCS2-mTORC1 signaling pathway is involved in the inhibition that decreases both mRNA and protein levels of the adipogenic transcription factor PPARγ which is required for adipocyte differentiation [200]. In our study, MC probably acts at the transcriptional level reducing Akt2 protein expression. Akt2 mediated phosphorylation of TCS2 protein which is associated with TCS1, acts as a critical negative regulator of the mTOR1. The loss of the TSC1-TSC2 function complex results in constitutive mTORC1 signaling through the mTORC1 dependent feedback mechanism and loss of mTORC2 activity that leads to a concomitant block of Akt2 signaling with its other downstream targets.

The results of both western blot and real-time quantitative PCR may be attributed due to down-regulation of transcription factors, SREBP 1 and PPARγ family members which slow down the de novo synthesis of fatty acids and triglycerides and inhibit the early differentiation of preadipocytes and adipogenesis in mature adipocytes.

In conclusion, our results demonstrate that Momordica charantia efficiently inhibits adipogenesis in 3T3-L1 adipocytes as evidenced by a significant reduction in intracellular lipid accumulation in a dose-dependent manner without showing apparent cytotoxicity. Furthermore, these suppressive effects of MC are possibly mediated by down-regulated expressions of PPARγ and SREBP1 mRNA and protein levels.
**Future prospects:** We plan to identify the bioactive compound(s) present in the 50% EEMC using high-performance liquid chromatography and mass spectrophotometry. The active compound, once isolated, will be re-tested on both the adipogenesis and adipolysis. The mechanism of action of EEMC on adipogenesis and adipolysis will be studied by Western blot analysis and RT-PCR assay. Further experiments will use primary human pre-adipocytes and animals to establish the therapeutic index of the purified active compound.