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RESEARCH ARTICLE

NEUROPROTECTIVE EFFECT OF POLYHERBAL FAST DISSOLVING TABLET OF CURCUMIN, QUERCETIN AND RUTIN AGAINST STREPTOZOTOCIN INDUCED DIABETIC NEUROPATHY IN RODANTS

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Key words:-
Curcumin, Diabetic Neuropathy, Hyperglycemic, Quercetin, Rutin

Abstract

Purpose: In the Current study we conducted the neuroprotective effect of the Polyherbal combination of curcumin, quercetin and rutin in Streptozotocin-induced Diabetic neuropathy in diabetic rat.

Method: In the 12 week study, two different formulations of polyherbal fast dissolving tablets were administered to diabetic rats. Neuropathic pain was assessed in diabetic rats with numerous aching trials and these tests were carried out for the consideration of thermal, mechanical, cold and hot hyperalgesia and nerve co-ordination. At the end of this study, trial animals were sacrificed and examined for biochemical parameters.

Result: Animals treated with poly herbal fast dissolving tablet to attenuate hyperglycaemia induced mechanical, thermal hyperalgesia and cold allodynia and improved the biochemical parameters in a dose-dependent manner.

Conclusion; From this it was conclude that polyherbal fast dissolving tablet of curcumin, quercetin and rutin significantly exhibits the antidiabetic, antioxidant and neuroprotective activities against streptozotocin-induced diabetic neuropathy in rats.

Introduction:-

Diabetes mellitus (DM) is a biological processing disorder categorized by hyperglycemia caused by indeficienct secretion of insulin secretion and abnormal action of insulin.[1] DM is a major health issue in developing and developed countries. According to International Diabetes Federation, approximately 470 million adults were living with diabetes; by 2045 it may be rise upto 700 million.[2]Abandoned diabetes leads to microvascular and macrovascular complications.[3] Diabetic neuropathy (DN) is the widespread type of microvascular obstacle of diabetes.[4]

Some drugs namely anticonvulsant and tricyclic antidepressants drugs are on hand for the treatment of neuropathic pain. Though, these drugs were stated to display an inclusive range of undesirable effects in the managing of neuropathic pain. So, there are a controlled figure of principle medicines for the treatment of diabetic neuropathic pain. Researchers, health care professionals are showing interest in alternative therapies for diabetic neuropathy.

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In the present study, we have chosen some flavonoids which are curcumin (Cm), quercetin (Qc) and rutin (Rt).Cm has a broad choice of pharmacological applications such as anti-inflammation, anti-human immune deficiency virus, anti-microbial, anti-oxidant, anti-parasitic, anti-mutagenic and anti-cancer[8-12] with low or no intrinsic toxicity. Rutin has significant scavenging properties on oxidizing species such as OH radicals, superoxide radicals, and peroxyl radicals. Therefore, it shows several pharmacological activities including anti-allergic, anti-inflammatory, and vasoactive, antitumor, antibacterial, antiviral, and anti-protozoal propertie.[13-14] Quercetin belongs to a sub-class of flavonoids known as flavonols, which find use in nutraceuticals or food supplements. Studies have shown that quercetin has antioxidant, anti-inflammatory, anti-bacterial, anti-coagulative, and anti-hypertensive properties.[15-20]

Experimental Section

Materials: The Polyherbal fast dissolving tablets (PHFDTs) were previously prepared from the direct compression method through the solid dispersion (SD) method. In the SD method, two polymers were used to determine the solubility enhancement of these herbal drugs.[21] The granules of PHT-I were prepared with PVPK30 and PHT-II were prepared with β-CD. In PHFDTs preparation,crospovidonewas used to increase the disintegration time of the tablet.[22] Due to this, they show their therapeutic effect very quickly and all the evaluation parameters were within the official limit and the solubility of these herbal drugs was increased. All the tablets were evaluated to determine their efficacy. All the parameters of the tablet were in official limit and drug release were also more than 70% within 5 minutes.

Acute and subacute toxicity study:

Acute and subacute toxicity studies of the polyherbal combination of Cm, Qc and Rt were previously determined inSwiss albino miceaccording to the OECD guidelines No.420 and 425 respectively. On the basis of previous toxicity studiesthe LD50 dose were selected in four doses of 250, 500, 1000 and 2000 mg/kg p.o.[23]

Drugs and Chemicals:

Duloxetine was purchased from Talent, India. Metformin was purchased fromGlycomet (USV) and Streptozotocin was purchased from M. P. BioMedicine, Mumbai, India and, all otherselected chemicals and reagents were of analytical grade.

Animals used:

Young and healthySD rats of either sex weighing between 180-250 gm werecollected from theanimal house facility fromCentral Drug Research Institute (CDRI), Lucknow. The animals were maintained under customary environmental conditions (23°C – 25°C, 12 h / 12 h light / dark cycle) and provided a standard pelleted diet (Dayal food Pvt. Ltd, Unnao) and water ad libitum. All the animals were habituated to the laboratory atmosphere for a week before the study proceeded. The experimental protocol for animal experiments was approved by the Institutional Animal Ethics Committee (IAEC) of Integral University, Lucknow (Uttar Pradesh) (Approval no. IU/IAEC/19/02).

Experimental plan:

In the current study, 7 groups were made with 5 rats in each group. In 6 groups diabetes were induced with a unit dose of STZ solution of 55 mg/kg b. w. In cold citrate buffer (PH 4.5, 0.01 M) and, administered intraperitoneally.[24] After 72 hrs, measured the blood glucose level of surviving rats and continue the further study with rats whose fasting blood glucose levels were above 250 mg/dl.[25]

The prepared poly herbal fast dissolving tablets and standard drugs were dissolved in distilled water and administered orally with the help of a gastric oral tube. Following groups were created with experimental animals;
Group-1: Normal control group (Distilled water 5 ml/kg, p.o)
Group-2: Diabetic control group (STZ 55 mg/kg, i.p)
Group-3: Diabetic group treated with Metformin (10 mg/kg, p.o)
Group-4: Diabetic group treated with Duloxetine (20 mg/kg, p.o)
Group-5: Diabetic group treated with PHC (each drug 50 mg/kg, p.o)
Group-6: Diabetic group treated with PHT-I (each drug 50 mg/kg, p.o)
Group-7: Diabetic group treated with PHT-II (each drug 50 mg/kg, p.o)

The study was performed for 12 weeks. The behavioral parameters and fasting blood glucose levels of experimental animals were determined on 0, 6th and, 12th week respectively. At the end of the 12th week, thiopental sodium (50 mg/kg i.p.) were administered to sacrifice the rats[26] and determined the analysis of isolated sciatic nerves for various

biochemical parameters. When animals were sacrificed and sciatic nerves were excised out carefully, immediately nerves were washed by using ice-cold saline (0.9% NaCl) along with 20mM EDTA to remove the blood, dried with tissue paper, weighed and were stored in 10% formalin solution, the stored nerves were used for histopathological examination.

Characterization of Behavioural Parameters:
Hyperalgesia and Allodynia:
Cold water tail immersion test:
In a cold water container (10^0C) the tail was immersed and observed the time duration for tail withdrawl and repeat the procedure for three times and calculate the mean value. For the prevention of tissue injury, a cutout time of 20 sec. should be maintained.

The decrease in tail contact time with cold water was observed as nociception, while increase in contact time was observed as an anti-allodynic effect.[27]

Hot water tail immersion test:
In a hot water container (55 ± 0.5^0C) the tail was immersed and observed the time duration for tail withdrawl and repeat the procedure for three times and calculate the mean value. For the prevention of tissue injury, a cutout time of 15 sec. should be maintained. Decrease in tail withdrawal time was observed as thermal hyperalgesia.[28]

Paw heat- hyperalgesia test (Eddy's hot plate method):
Heat nociceptive threshold was an index of thermal hyperalgesia. Preheat the plate and maintained the temperature of 52.5±2.0°C and placed the rats on the hot plate and note down the time for the nociceptive threshold, licking and jumping of the hind paw and maintain the cutoff time was at 20 sec.[29]

Mechanical hyperalgesia (Pinprick test):
A bent gauge needle was touched on injured hind paw at 90° and then noted the duration of the paw withdrawal in seconds by maintaining the 20 sec as cut-off time.[30,31]

Cold hyperalgesia (Acetone drop test):
It is a modified method for the the estimation of thermal sensitivity as marked out by Choi Y.[32] Rats were placed in a new metal mesh cage for 20 minutes to habituate and acclimatise. Appied the acetone drop (50µL), gently onto the hind paw. A quick cold chemical sensitive reaction was generated and then it was considered as nociceptiveresponse.

Motor coordination:
The rotarod apparatus was used to conduct this parameter, rats were placed on a rotating spindle (25rpm). The test was performed for 5 minute and noted the falling time of each rat from the rotating spindle.[33]

Spontaneous Locomotor (Exploratory) Test:
This parameter was used to observe the exploratory behaviour of the rats by using actophotometer. Observe the each animal for a period of 5 min in a closed area having 6 photocells on the external wall. Interruptions of photocell beam (locomotor/exploratory action) of rats were recorded by digital counter.[34,35]

Estimation of Biochemical Parameters:
Blood glucose levels: Observation of the blood glucose levels was completed with glucose-oxidase principle[36] at the interval of days 0 and 3, weeks4, 8, and 12 weeks by puncturing the lateral vein of the rat's tail by using a glucometer (Accu-Chek sensor from Roche Diagnostic Corporation).[37] At the end of the study, rats were terminated with 50 mg/kg thiopental sodium i.p injection[38] and sciatic nerve was isolated and estimate lipid peroxidation (TBARS), SOD, total protein and total calcium levels.[39-41]

Histopathological Assessment
On the last day of the study before collecting blood samples, the animals were sacrificed after anaesthetizing the animals with thiopental sodium, sciatica nerves were dissected out washed with normal saline and weighed. The nerves were kept in 10% formalin solutions. The total gross examinations of the nerves were performed and visible superficial, morphological and anatomical abnormalities were recorded. Later the organs were embedded in paraffin

wax, sliced into a 4 µm section and further stained by using Hematoxylin and Eosin. Under the light microscope, the photomicrograph was obtained for histopathological evaluation in this study (Mshot MD30-B). The analysis was performed by an experienced pathologist (Alpine diagnostic, Lucknow) unaware of different treatment groups as coding of each nerve was done.

Statistical analysis:

The results were expressed as Mean±SD. The intergroup variation was measured by using One-way analysis of variance (ANOVA) followed by Bonferroni t-test. The statistical analysis was done by using SigmaStat 3.5.Values of $P < 0.05$ were considered statistically significant.

Results:-

Hot & Cold water tail immersion test:

PHFDTs treated rats were showed the early significant improvement in tail withdrawal latency at dose 50 mg/kg from 0th week and remaining doses of each herbal drug 50 mg/kg were showed the effect on 6th week for hot and **(Fig.1)** cold **(Fig.2)** water tail immersion.

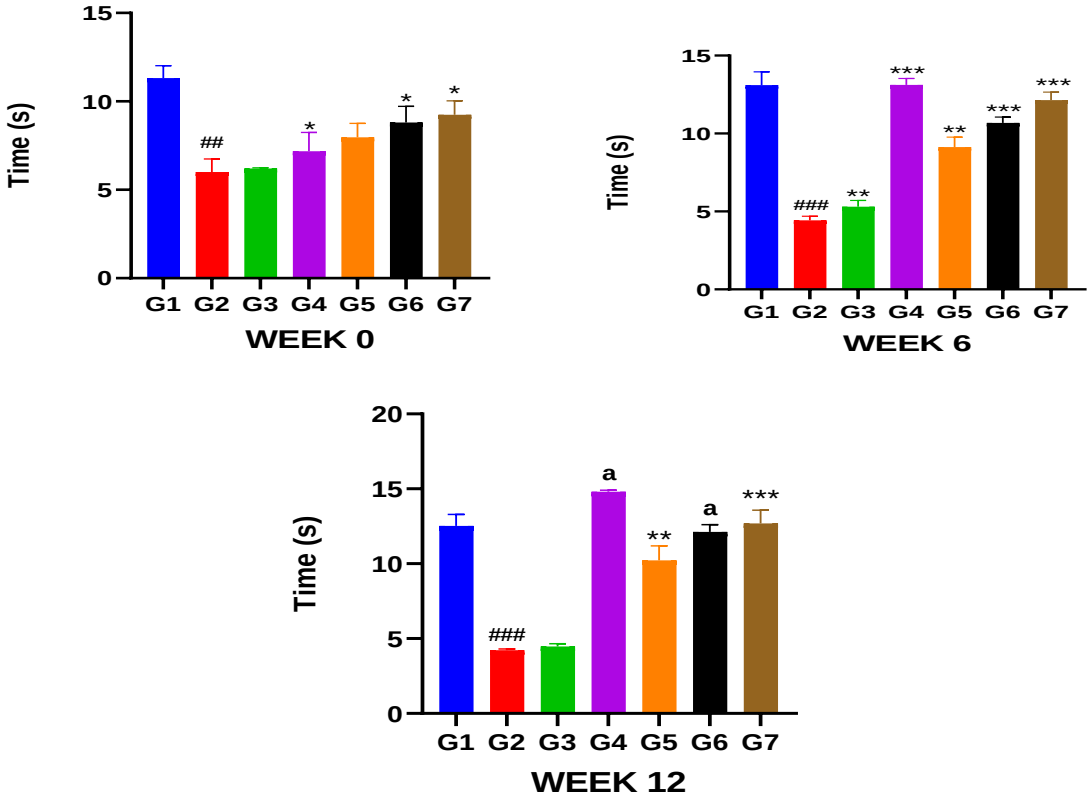


Figure 1:- Graph of Coldwater tail immersion test. Values were depicted as means ± SD for 5 SD rats per group. Statistically significant differences between groups were measured using one-way ANOVA with a nonparametric paired t-test. Where ##$p < 0.01$, slightly significant, ###$p < 0.001$, very significant, when the values of normal control group (G1) compared with disease control group (G2). Where $p>0.05$, non-significant, *$p < 0.05$, significant **$p < 0.01$, slightly significant, ***$p < 0.001$, very significant; =ap<0.0001, highly significant, when the values of all the remaining treatment groups were compared with disease control group (G2).

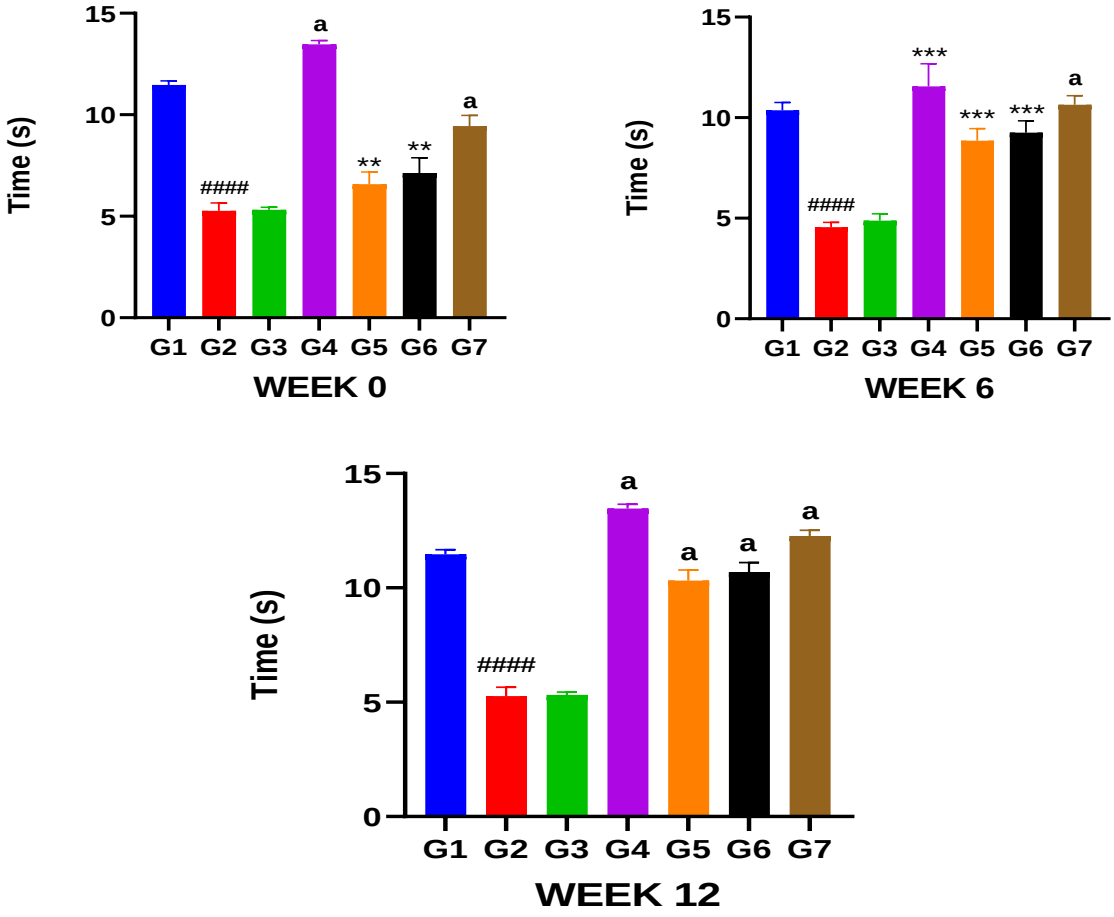


Figure 2:- Graph of Hot water tail immersion test. Values were depicted as means ± SD for 5 SD rats per group. Statistically significant differences between groups were measured using one-way ANOVA with a nonparametric paired t-test. Where ####p < 0.001, highly significant; when the values of the disease control group (G2) was compared with the normal control group (G1). Where p>0.05, non-significant, **p < 0.01, slightly significant, ***p < 0.001, very significant; =ap<0.0001, when the values of all the remaining treatment groups were compared with disease control group (G2).

Paw heat- hyperalgesia test (Eddy's hot plate method):

There was a significant difference in the reaction time for nociceptive stimuli in the normal (G1) and diabetic group during the treatment period. Treatment with Duloxetine (20 mg/kg) showed a significantly improvement the paw withdrawal latency from the 6th week. Whereas, pure drugs 50 mg/kg each were showed a significantly improvemnent the paw withdrawal latency from the 12th week in comparison with disease control rats and PHFDTs 50mg/kg were significantly improved the paw withdrawal latency from the 0^{th} week **(Fig.3).**

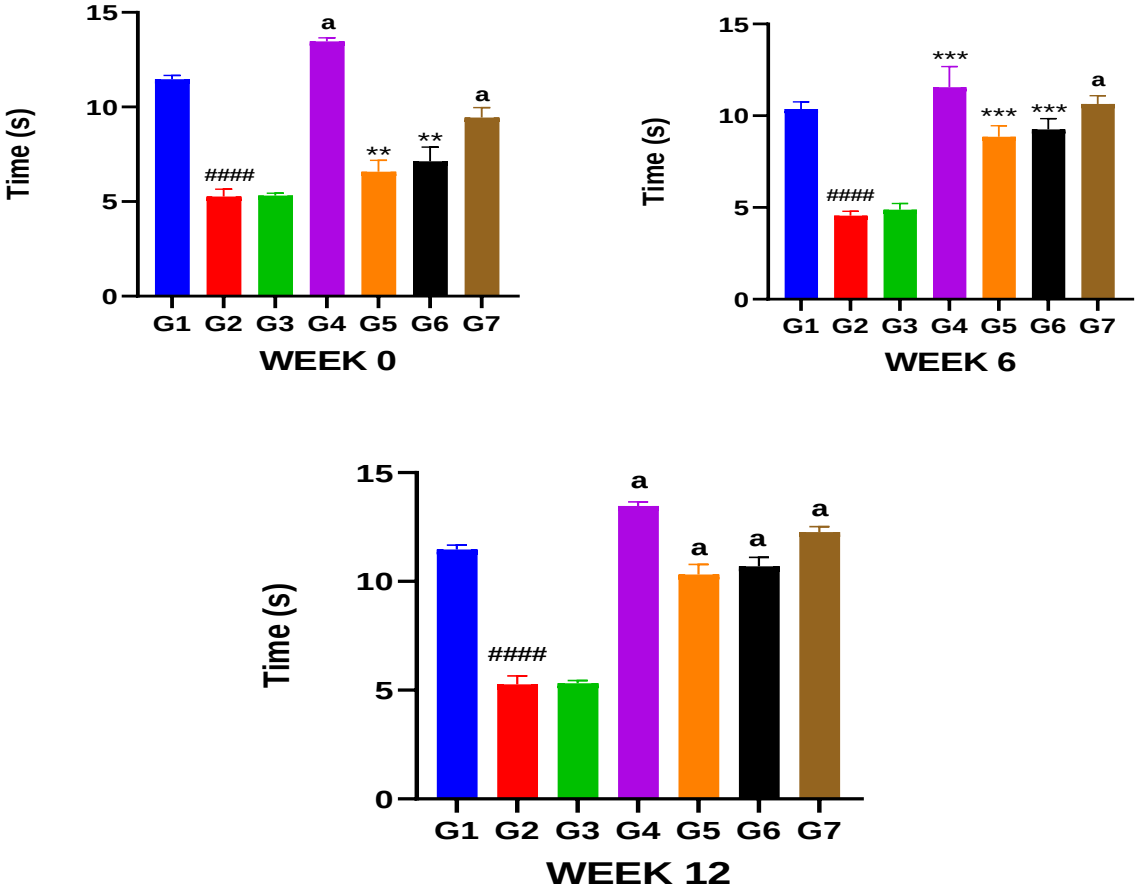


Figure 3:- Graph of Paw heat-hyperalgesia test. Values were depicted as means ± SD for 5 SD rats per group. Statistically significant differences between groups were measured using one-way ANOVA with a nonparametric paired t-test. Where, ##p < 0.01, slightly significant, ####p < 0.001, highly significant; when the values of disease control group (G2) was compared with normal control group (G1). Where p>0.05, non-significant, *p < 0.05, significant, **p < 0.01, slightly significant, ***p < 0.001, very significant; =ap<0.0001, highly significant, when the values of all the remaining treatment groups were compared with disease control group (G2)

Mechanical hyperalgesia (Pinprick test):

A hyperrresponsiveness to an injurious stimulus was observed with a significant rise in paw withdrawal latency in disease control as compared with normal control. Treatment with a pure herbal drug (50 mg/kg, each) used significant reduction from the 6th week in paw withdrawal latency in a dose-dependent manner. The anti-nociceptive effect of PHFDTs have parallel effects to the, duloxetine**(Fig.4)**.

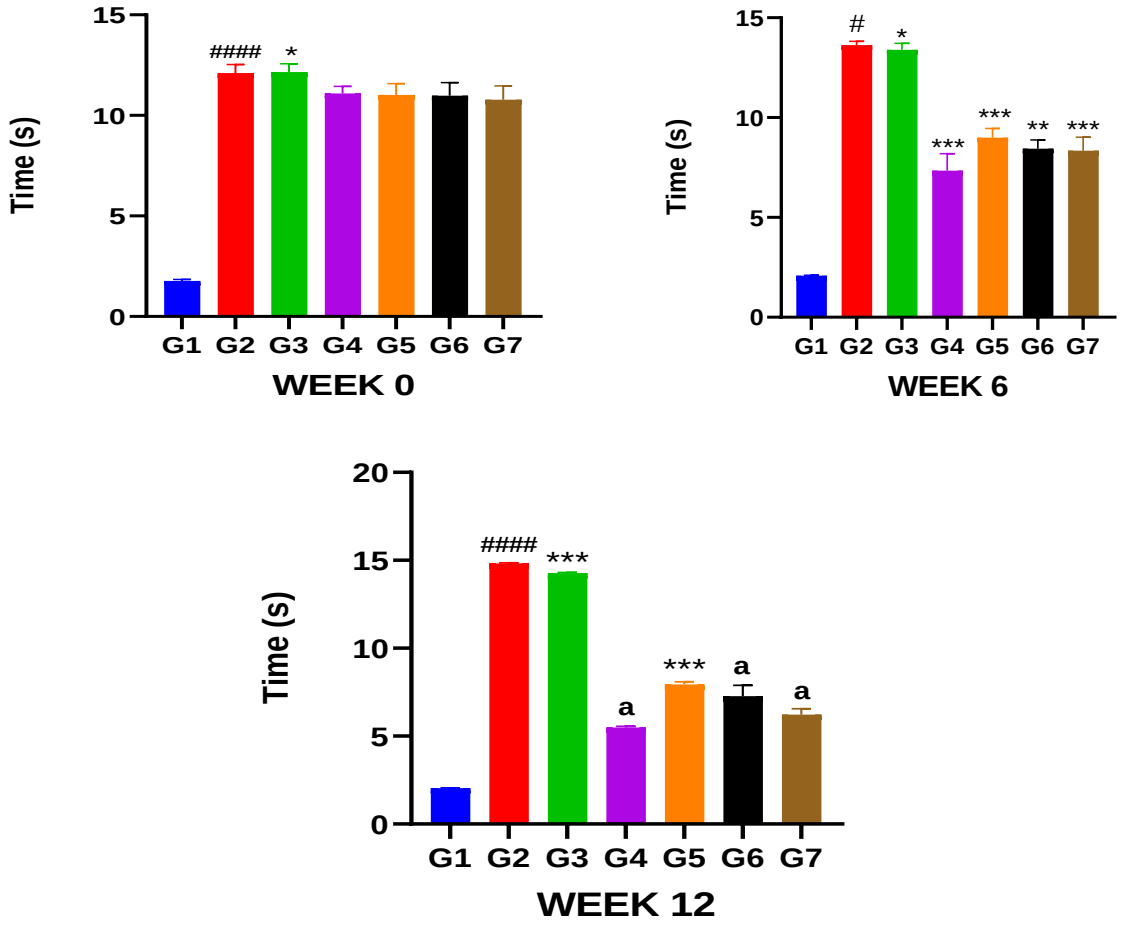


Figure 4:- Graph of Mechanical hyperalgesia test. Values were depicted as means ± SD for 5 SD rats per group. Statistically significant differences between groups were measured using one-way ANOVA with a nonparametric paired t-test. Where, #p < 0.05, significant, ####p < 0.001, highly significant; when the values of disease control group (G2)was compared with normal control group (G1). Where p>0.05, non-significant, *p < 0.05, significant, **p < 0.01, slightly significant, ***p < 0.001, very significant; =ap<0.0001, highly significant, when values of all the remaining treatment groups were compared with disease control group (G2).

Cold hyperalgesia (Acetone drop test):

In this test, applying acetone on the plantar surface of hyperglycemicrats results in cold allodynia, which is implicated as a rise in paw withdrawal interval than the normal control (G1). PHFDTs (50 mg/kg) treated aniamls were showed significant results from the 0th week, and pure herbal drug 50 mg/kg each were started to a shown reduction in paw withdrawal latency from 6th week as compared to disease control (G2). **(Fig.5).**

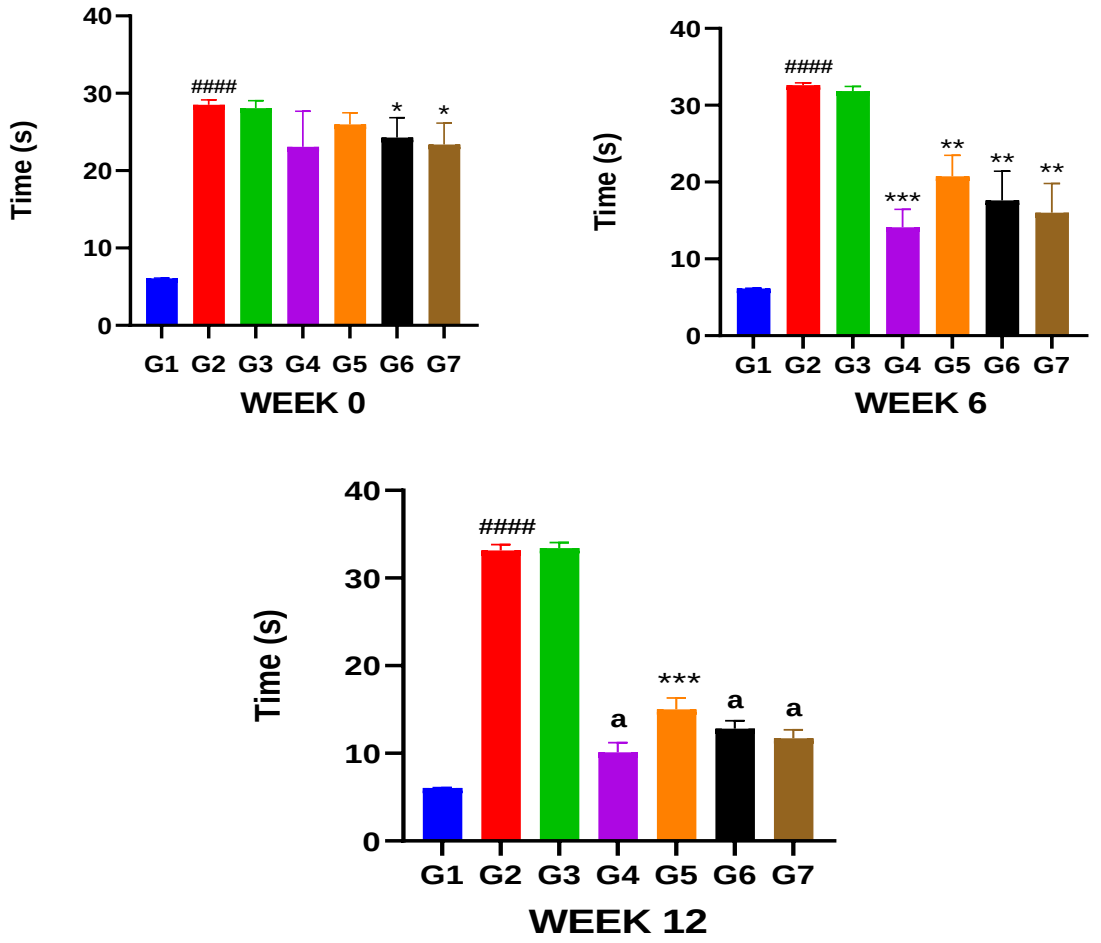


Figure 5:- Graph of Cold hyperalgesia test. Values were depicted as means ± SD for 5 SD rats per group. Statistically significant differences between groups were measured using one-way ANOVA with a nonparametric paired t-test. Where, ####p < 0.001, highly significant; when the values of the disease control group (G2) was compared with the normal control group (G1). Where p>0.05, non-significant, *p < 0.05, significant, **p < 0.01, slightly significant, ***p < 0.001, very significant; =ap<0.0001, highly significant, when the values of all the remaining treatment groups were compared with disease control group (G2).

Motor coordination locomotor activity:

Rats treated with PHFDTs were shown significant improvement in muscle grip strength and locomotor activity as compared with disease control. Improvement in motor coordination activity was achieved with the PHFDTs 50mg/kg from 0th **(Fig.6)**. Whereas, PHFDTs50 mg/kg were significantly improved the locomotor activity from 0th week**(Fig.7)**.

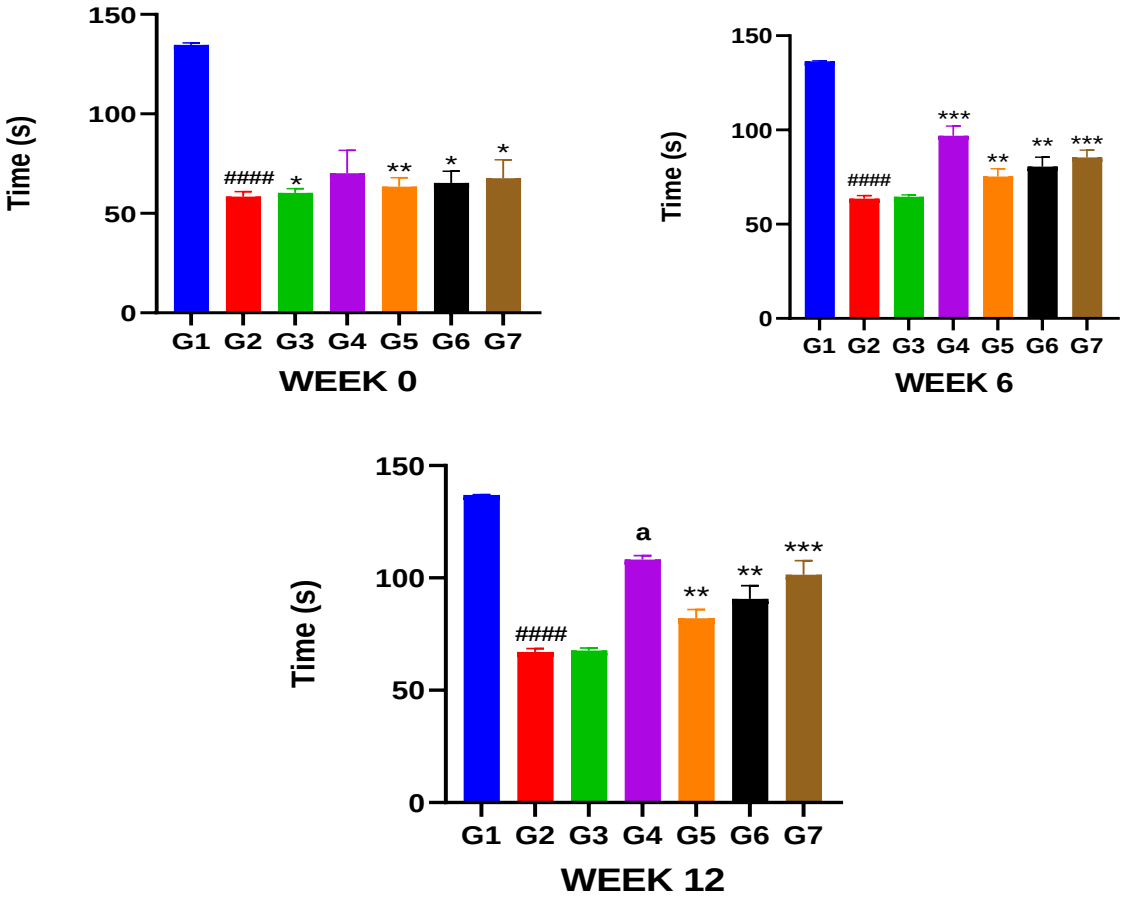
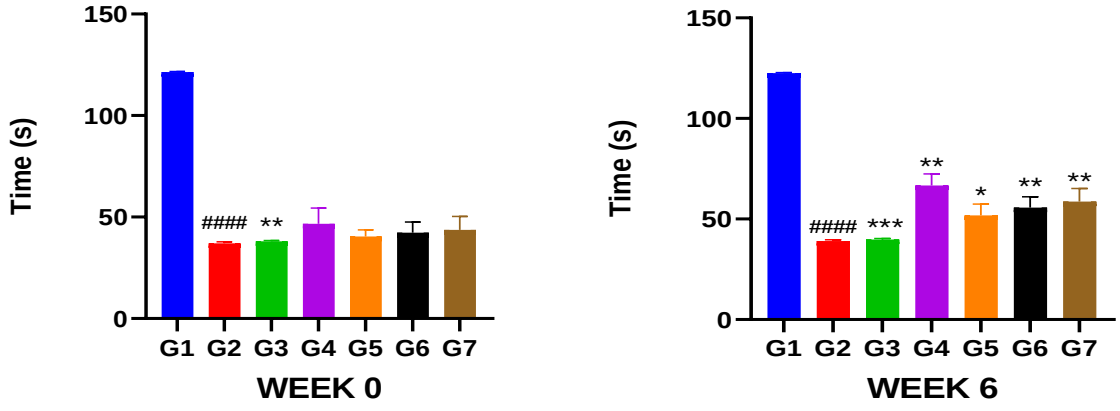


Figure 6:- Graph of Motor coordination test. Values were depicted as means ± SD for 5 SD rats per group. Statistically significant differences between groups were measured using one-way ANOVA with a nonparametric paired t-test. Where, ####p < 0.001, highly significant; when the values of the disease control group (G2) was compared with the normal control group (G1). Where p>0.05, non-significant, *p < 0.05, significant, **p < 0.01, slightly significant, ***p < 0.001, very significant; =ap<0.0001, highly significant, when the values of all the remaining treatment group was compared with disease control group (G2).



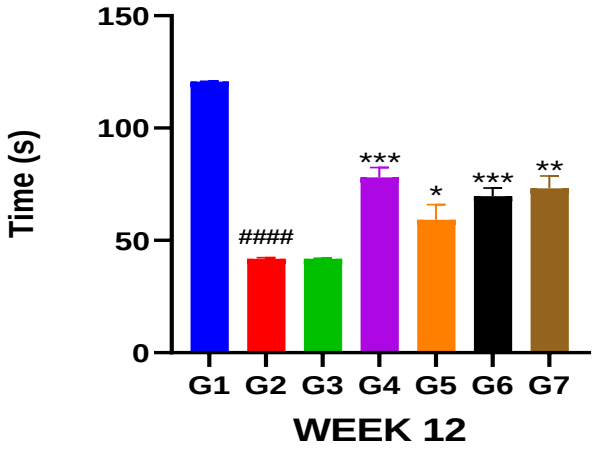
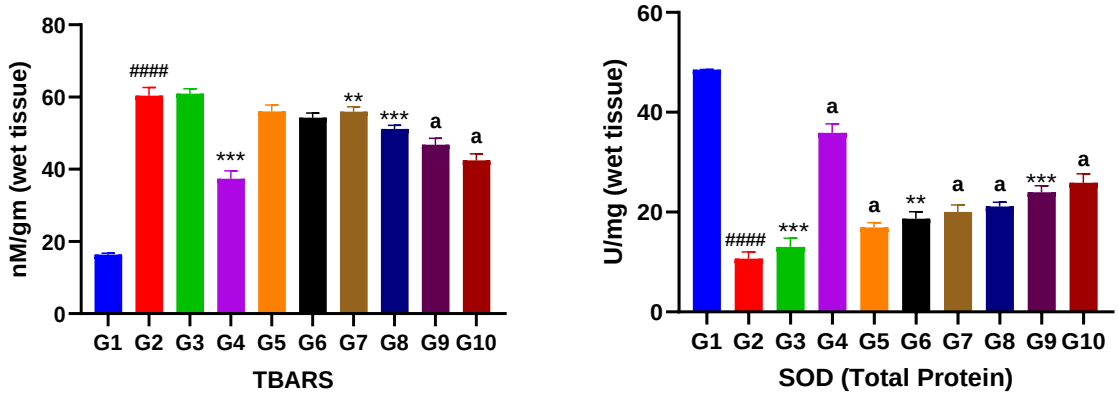


Figure 7:- Graph of Spontaneous locomotor test. Values were depicted as means ± SD for 5 SD rats per group. Statistically significant differences between groups were measured using one-way ANOVA with a nonparametric paired t-test. Where, ####p < 0.001, highly significant; when the values of the disease control group (G2) was compared with the normal control group (G1). Where p>0.05, non-significant, *p < 0.05, significant, **p < 0.01, slightly significant, ***p < 0.001, very significant; =ap<0.0001, highly significant, when the values of all remaining treatment groups were compared with diseasecontrol group (G2).

Biochemical parameters:

At the end of the study, STZ induced diabetic rats showed elevated levels of lipid peroxidation, total calcium and a decrease in SOD levels and total protein value when compared with normal control **(Figure 8)**. The PHFDTs 50 mg/kg and duloxetine 20 mg/kg treated rats were significantly reduced the elevated lipid peroxidation levels, SOD, total protein levels and in total calcium levels during 12 weeks of treatment as compared with G2.



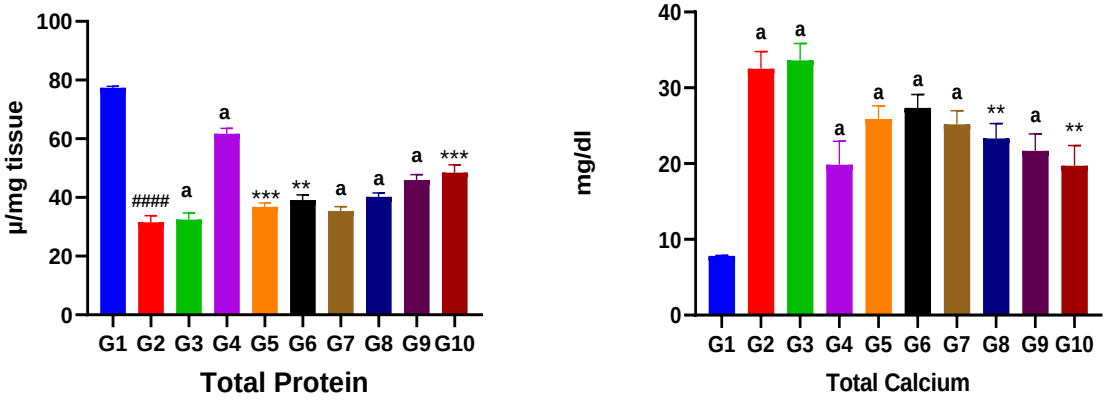


Figure 8:- Graph of the biochemical parameter. Values were depicted as means ± SD for 5 SD rats per group. Statistically significant differences between groups were measured using one-way ANOVA with a nonparametric paired t-test. Where, ####p < 0.001, highly significant; when the values of the disease control group (G2) was compared with the normal control group (G1). Where p>0.05, non-significant, **p < 0.01, slightly significant, ***p < 0.001, very significant; =ap<0.0001, highly significant, when the values of all the remaining treatment group were compared with disease control group (G2)

Assessment of blood glucose level

A noticeable rise in fasting blood glucose levels was observed in STZ treated groups as compared to normal control. Whereas, treatment with PHFDTs 50mg/kg and metformin 10mg/kg caused a significant reduction in the fasting blood glucose level in a dose-dependent manner. While duloxetine have not any remarkable effect on the blood glucose levels in the entire study period **(Figure 9)**.

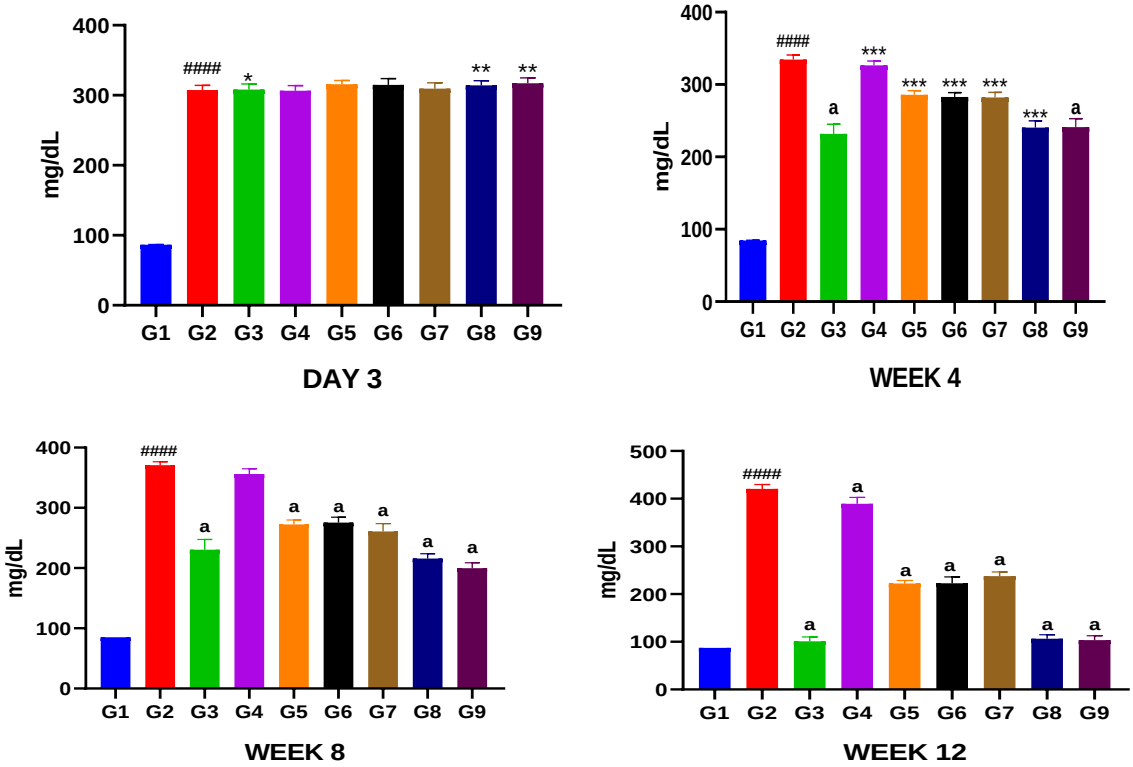
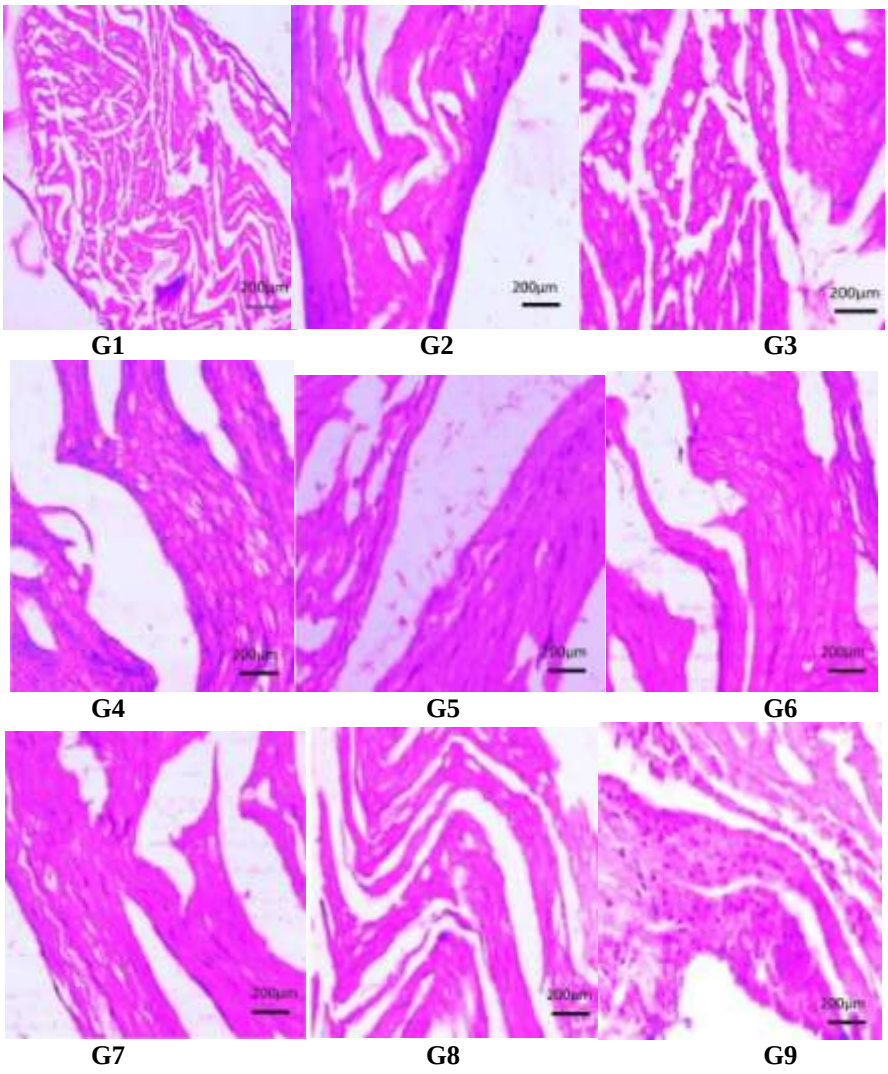
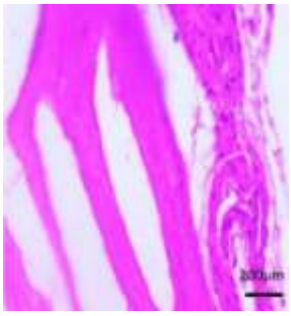


Figure 9:- Graph of Blood glucose level. Values were depicted as means ± SD for 5 SD rats per group. Statistically significant differences between groups were measured using one-way ANOVA with a nonparametric paired t-test. Where, ####p < 0.001, highly significant; when the values of the disease control group (G2) was compared with the normal control group (G1). Where p>0.05, non-significant, *p < 0.05, significant, **p < 0.01, slightly significant, ***p < 0.001, very significant; =ap<0.0001, highly significant, when the values of all the remaining treatment group were compared with disease control group (G2)

Histopathological Studies

Photomicrographs of rat sciatic nerve of the normal control group show, minimum interstitial tissue, long spindle-shaped vascular nuclei, myelin sheath well marked, and minimum fibro-fatty tissue. There were no neuronal hypertrophy or evidence of necrosis and/or round cell infiltrates in normal control group rats. The photomicrographs of polyherbal combination (PHT-12 &PHT-24) and Duloxetine treated groups showed healthy nerve fibre with minimal necrotic infiltrations.**(Figure: 10)**





G10

Figure 10:- The histopathology of the sciatic nerve from the different groups of SD rats (40 × magnification; Scalar bar: 100 μm). No significant damage (ex. neuronal necrosis, neuronophagia, cellular necrosis, intact glial cells) was observed in the sciatic nerve cells of any treated group rat. The architecture of sciatic nerve cells is normal in all experimental groups when compared to the normal control group (G1).

Statistical analysis:

The results were signified as means ± standard deviation (SD). The quantitative statistical analysis was performed by using the statistical analysis software GraphPad prism 6.0. The differences between the treatment group and normal control groups were identified by applying analysis of variance (one-way ANOVA) followed by the Dunnet test, the nonparametric paired t-test was performed to compare a couple of variables and obtain the significance level. Significance was measured at values of $p < 0.05$. A value of $p > 0.05$ was considered nonsignificant.

Discussion:-

In this study, PHFDTs were given for prevention as well as treatment of neuropathic pain in STZ induced diabetic rats. The development of consistent with previous reports. STZ gain popularity to induce type-1 hyperglycemia in rodents which resemble human insulin-dependent diabetes mellitus.[42] STZ induced hyperglycemia probably due to pancreatic DNA alkylation through GLUT2 transporter mechanism[43], which intern triggers multiple biochemical pathways such as polyol pathway, hexosamine pathway, PKC, AGE product, and PARP pathway all of these pathways contribute towards oxidative stress by generating ROS in mitochondria results in nerve damage and neuropathy.[44]

In this study, diabetic rats showed a significant rise in blood glucose levels than normal rats. PHFDTs (150 mg/kg b.w) treated hyperglycaemic animals were shown a significant reduction in blood glucose levels throughout the experiment. STZ induced diabetic animals are the models for chronic neuropathic pain with hyperalgesia and allodynia that reflect symptoms observed in diabetics.[45]

Hence, the behavioral parameters such as thermal and cold hyperalgesia; and allodynia were assessed by using a hotplate, pinprick, acetone drop test, and hot and cold water tail immersion tests along with motor coordination as well as locomotor activity. In the behavioral examination, diabetic rats were shown a significant reduction in tail and paw withdrawal latency than the normal control rats, which is an indication for the decreased nociceptive threshold to heat resulting in hyperalgesia and allodynia.

Similar models of thermal hyperalgesia and tail-flick latency have been reported previously in STZ induced diabetic animals. The delay in tail withdrawal response depicts the involvement of spinal reflex arc and the delay in paw withdrawal latencies to noxious thermal stimuli depicts the involvement of supraspinal sensory pathways. The hyperalgesic response to a noxious stimulus (pinprick) and development of cold chemical sensitivity in diabetic rats was shown a significant rise in hind paw withdrawal latency than the normal rats. It has been reported that the involvement of TRPA1 and ATP-gated purinergic ionchannel P2X3 may be responsible for mechanical hyperalgesia.[46] However, rats treated with PHFDTs 150 mg/kg were shown improvement in muscle grip strength at 0th and 6th weeks respectively as well as progress in locomotor activity was observed at 6th week with 150 mg/kg. PHFDTs alleviate hyperglycemia-induced mechanical, thermal hyperalgesia, and cold allodynia. It might be due to straight glycaemic control reverses the hyperglycemia-induced generation of ROS, which intern involved in the regulation of gene promoting inflammatory reaction results in neuronal dysfunction and generation of pain. Moreover, it is well established that ROS are gravely involved in pain transmission.[47]

The present study shows a significant rise in lipid peroxidation, and decreased nerve protein, superoxide dismutase levels are the indication of the involvement of oxidative stress in diabetes-induced neuropathy. Generation of peroxynitrite by a reaction between superoxide anions and nitric oxide results in protein nitrosylation, lipid peroxidation, DNA damage, and cell death shows direct toxic effects on nerve tissue.[48] SOD protects biological tissues from highly reactive superoxide anions by converting them to hydrogen peroxide, this hydrogen peroxide intern converted to water with the help of reduced GSH, hyperglycemia is known to involve in nonenzymatic glycosylation which results in reduced activity of SOD in the sciatic nerve of animals. Thus, the concurrent decrease in the endogenous antioxidant defense system makes sciatic nerves more vulnerable to hyperglycemia-induced oxidative stress. Chronic treatment with PHFDTs significantly increases tissue SOD levels and reduces lipid peroxidation in diabetic animals.[49]

The present study showed a significant rise in intracellular calcium concentration in neuropathic rats when compared with disease control rats. Excess calcium will participate in triggering a response of calpain and calmodulin and calcium-dependent kinases lead to imbalanced homeostasis in the nervous system result in neuronal hyperexcitation [50]. Chronic treatment with PHFDTs significantly blocks the calcium conduction in the nerve tissue and alleviates hyperglycemia-induced neuropathy.

Conclusion:-

On the basis of data in hand, it was concluded that polyherbal combination produced an ameliorative effect in diabetic neuroopathy that may be attributed to its herbal constituents' multiple effects, such as antioxidant, neuroprotective, antidiabetic, antistress, hepatoprotective, antifibrinolytic, and anti-inflammatory activity. Therefore, the fast dissolving polyherbal combination comprising curcumin, quercetin and rutin phytoconstituents was found to have antidiabetic and antioxidant effects and play a prominent role in attenuation of diabetes induced neuropathy.

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Institutional Review Board Statement:
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The authors declare no conflict of interest amongst each other

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