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INVESTIGATIONS ON *ONOSMA HISPIDUM* WALL ROOT EXTRACTS FOR *IN-VITRO* ANTIDIABETIC, PROLIFERATIVE AND CYTOTOXIC EFFECTS

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ABSTRACT

The aim of this study was to investigate the impact of various extracts of roots of *Onosma hispidum* for anti-diabetes, proliferative and cytotoxic effects using *in-vitro* assays. Phytochemical screening of powder and extracts was done by employing standard methods. Anticancer activity was assessed by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and short term proliferation assay on HepG2 cell line. The antidiabetic potential was determined by using alpha-amylase inhibition assay and non-enzymatic glycosylation of haemoglobin assay. Data suggested that primary (CHO; 68.06 ± 0.19 and Proteins; 9.48 ± 0.27) and secondary metabolites (mg/g); max. Flavonoids (47.55 ± 0.13) in aqueous extract, max. Polyphenols (107.77 ± 0.57) in Pt. ether extract and max. glycosaponins (94.5 ± 0.51) in chloroform extract, exhibited variations among various extracts obtained from roots of *Onosma hispidum*. Petroleum ether and aqueous extracts exhibited significant impact ($p<0.05$) on the proliferation of HepG2 cell lines at different time points compared to control, while in case of MTT assay, no significant results($p>0.05$) were obtained after overnight incubation. Improved antidiabetic activity was exhibited by petroleum ether extract (70% inhibition) as compared to Acarbose standard using alpha-amylase inhibition assay, with an IC_{50} of 80µg/ml. In non-enzymatic glycosylation of haemoglobin assay, better potential was shown by chloroform extract with an $IC_{50}$of 35µg/ml, when compared to a standard - Tocopherol. This study revealed that the roots of *Onosma hispidum* possess nutritional value and antidiabetic properties, along with significant impact on cell (HepG2) proliferation.

Keywords: *Onosma hispidum*, *In-vitro*, Polyphenols, Antidiabetic, Anticancer.

INTRODUCTION

In recent years, researchers paid considerable attention to the naturally occurring sources of medicines, which serve as traditional remedies for various ailments and conditions (Bako *et al.*, 2005). It has been estimated that in developing countries more than 80% of the population meet their health care needs using herbal medicines (WHO, 2002).The reason behind the extensive use of herbal medicines may be due to general beliefs that in comparison to allopathic medicine, herbal medicines are easily available, cheaper, pose little risk of toxicity with improved safety profiles duly endorsed by cultural and social beliefs of the people regarding herbs (Gurav *et al.*, 2011). Plants serve as a natural source of vital phytochemicals such as alkaloids, tannins, flavonoids, glycosaponins, polyphenols and terpenoids, which are produced by the plants as secondary metabolites (Sher, 2009). These secondary metabolites are responsible for various beneficial effects rendered by plant constituents, namely, flavonoids and polyphenols, which may serve as antioxidant, thus can contribute towards anti-carcinogenic and anti-mutagenic effects (Urquiaga and Leighton, 2000; Okwu, 2004).

Onosma hispidum, a perennial herb with a sharp and bitter taste has been reported to be used traditionally as an important source of red dye yielding root "Ratanjot", which is used for coloring oils, food stuffs and other preparations (Khanna and Dedhia, 1999).Medicinally, the plant has cooling, laxative, alexipharmic and anthelmintic effects and is also used to treat various diseases of eye, blood, bronchitis, wounds, bites and abdominal pain (Ahmad *et al.*, 2003). The ethanol extract of the whole herb is reported to have inhibitory activity against cholinesterase and lipoxygenase enzymes (Ahmad, 2006). Naz *et al.* (2006) investigated root bark ethanol extracts of *Onosma hispidum* for anti-tussive and antibacterial activity. Kumar *et al.* (2010) reported the hypoglycemic potential of methanol extract of roots of *Onosma hispidum* by testing it in glucose-loaded, normoglycemic and alloxan-induced hyperglycemic rats.

Hepatocellular carcinoma (HCC) is one of the most fatal cancers with high prevalence among the developing countries of Asia and Africa (Darvesh *et al.*, 2012). It is currently ranked as the fifth most frequent

carcinoma and a third leading cause of deaths, worldwide (Jemal *et al.*, 2005). According to Wu *et al.* (2009), owing to the availability of limited treatment options and poor prognosis associated with hepatic cancers, phyto-pharmaceuticals have drawn considerable attention of health care providers to use phytochemicals as adjuvant therapy to improve the survival rate and quality of life. Thus, there is a pressing need to find new drug sources that may be effective in the control of life threatening diseases. For this purpose, plants serve as a rich source of phytochemicals with potential antioxidant properties which may provide a suitable and affordable alternative for the alleviation of HCC (Darvesh *et al.*, 2012). In the quest of finding potential anticancer agents from different herbs, these herbs have been tested for their cytotoxic effects using mammalian cancer cell lines before testing their *in vivo* anti-cancer potential(Houghton *et al.*, 2007). Due to this, various herbs are being tested using HepG2 cell line derived from human hepatocellular carcinoma cells obtained from liver tissues (Tavakkol-Afshari *et al.*, 2008).

Likewise, diabetes, a multifarious endocrine disorder typified by chronic hyperglycemia with concomitant disturbances in proteins, fats and carbohydrate metabolism due to absolute or relative insulin secretion or action. Globally, the number of diabetic patients is predicted to reach 366 million by the year 2030 (Patel *et al.*, 2012). More than 1% of the world's population is thought to be affected with diabetes and their number is increasing day by day. According to Wild *et al.* (2004) Pakistan is ranked 7th among the countries having highest burden of diabetes mellitus, and the number of people with diabetes is expected to increase from 5.2 million to 13.2 million by the year 2030. Despite the role of various antidiabetic agents in managing the disease, insulin and oral agents have several limitations (Christie and Levy, 2013), thus new drugs with improved safety and efficacy are need of the hour. Recently, there is an increasing interest of finding natural remedies to replace the synthetic ones in order to get better outcomes (Middha *et al.*, 2012).Additionally, synthetic drug molecules have been accused of potentially the primary source of antibiotic resistance, yet surprisingly, natural drug sources, involving various plant extracts in combination with antibiotics, have been suggested as a mainstay therapy that might prevent the reemergence of infections caused by antibiotic resistant organisms. So, natural products are playing a pivotal role in combating infections that would, otherwise, be difficult to control by synthetic agents only (Hemaiswarya *et al.*, 2008).Hasani-Ranjbar *et al.* (2008) emphasized that the natural source of medications with pronounced anti-diabetic activity with fewer side effects are becoming the major focus of researchers due to poor management and lack of proper treatment of disease.

To the best of our knowledge, no work is being carried out to investigate the anticancer and *in-vitro* anti-hyperglycemic effect of roots of *Onosma hispidum*. Therefore, the present work is undertaken to evaluate the anticancer and anti-hyperglycemic potential of extracts of *Onosma hispidum* roots in different solvents employing *in-vitro* anticancer and antidiabetic models.

MATERIALS AND METHODS

Plant Material: About 3 kilograms of the dried roots of *Onosma hispidum* were purchased from a local vendor of Paparmandi bazaar, Lahore. For authentication, botanical identification was carried by an expert Taxonomist of Botany Department, Government College University (GCU), Lahore, Pakistan, using relevant microscopic and manual procedures, where a voucher specimen was also deposited under the reference number GC. Herb. Bot. 2938.

Solvents and Chemicals: Petroleum ether (Sigma, USA), Chloroform (May and Baker, UK), Methanol (Merck, Germany), Ethanol (Merck, Germany), Deionized water, n-hexane (Merck, USA), Hydrochloric acid (BDH, England), Sulphuric acid (BDH, England), Nitric acid (BDH, England), Sodium carbonate (Merck, Germany), Copper sulphate (Merck, Germany), Potassium acetate (Merck, Germany), Aluminium nitrite (Merck, Germany), Acetone (Merck, USA), Quercetin(Sigma, USA), Bovine serum albumin(Sigma, USA), Gallic acid (Sigma, USA), Anhydrous glucose (Merck, Germany), Triton X (Sigma, USA), Folin Ciocalteu's Phenol Reagent (Unichem Chemicals, India), Anthrone Reagent (Sigma, USA), Sodium hydroxide (Merck, Germany), Sodium chloride (Merck, Germany), Sodium hydrogen phosphate (BDH, England), Disodium hydrogen phosphate (Riedel-de Haen, Germany), α-Amylase (Unichem Chemicals, India), 3, 5-dinitro salicylic acid (BDH, England), Potassium sodium tartrate (BDH, England), Distilled water, Starch, Acarbose tablets (Glucobay 500, Bayer), Haemoglobin (HIMEDIA Laboratories Pvt. Ltd), Gentamycin (Howards Pharmaceuticals), Phosphoric acid(Sigma, USA), Tocopherol (Fluka BioChemika), Dimethyl sulfoxide (DMSO) (Daejung, Korea), Penicillin – Streptomycin solution (Hyclone, USA), Fetal bovine serum (FBS) (Hyclone, USA), Dulbecco's modified Eagle medium (DMEM) (Hyclone, USA), Phosphate buffer saline (PBS) (Oxoid, England), MTT Reagent (Bio world, Dublin), Trypsin (Hyclone, USA).

Preparation of Plant Extract: After drying, extraction was carried out using two methods, hot and cold as described previously (Islam *et al*., 2015). Hot extraction was performed by using Soxhlet extractor by packing 175grams of root powder in the filter paper and extracted sequentially with various solvents namely, petroleum

ether, chloroform and methanol. Cold extraction was performed by macerating 25 grams of powdered material using ethanol and distilled water as solvents.

Proximate Analysis: The moisture content, total ash, acid insoluble, water soluble and sulphated one, and extractive values, alcohol and water soluble, were determined according to standard procedure (USP 2005).

Phytochemical Screening

Estimation of Primary Metabolites: The powdered plant material was analyzed for the estimation of total lipids, total proteins and total carbohydrates by the methods described by Besbes *et al*. (2004), Lowry *et al*. (1951) and Al Hoti *et al*. (1998) respectively.

Estimation of Total Lipids Content: Seventy five grams of root powder was extracted with n- hexane in soxhlet extractor. The extract was dried in a tarred flask using rotary evaporator at 40ºC. Total lipid content was determined by weighing the dried extract. The total lipid content was expressed as mg/g of the powder taken (Besbes *et al*., 2004).

Estimation of Total Protein Content: About one gram of the dried root powder was weighed and mixed with 10 ml of distilled water and 5 drops of triton-X in a centrifuge tube, after 30 minutes centrifuged at 2700 rpm for 10 minute and supernatant was collected. In 100µl of the supernatant volume was made 1ml with distilled water and 3ml of reagent C which was prepared by mixing 50ml of reagent A (2% sodium carbonate in 0.1N sodium hydroxide) and 1ml of reagent B (0.5% copper sulphate in 1% potassium sodium tartrate) and 0.2ml of FC reagent was added. After incubation, absorbance was measured at 600nm using BSA- Fraction V as standard (Lowry *et al.*, 1951).

Estimation of Total Carbohydrates: Formula described by Al-Hooti *et al.* (1998) was used to quantify the total carbohydrate contents.

$$\text{Total carbohydrate} = 100 - (\text{total moisture} + \text{total ash} + \text{total lipids} + \text{total proteins})$$

Estimation of Secondary Metabolites: The extracts of roots were subjected to analysis for the estimation of total polyphenols, total flavonoids, total polysaccharides and total glycosaponins by adopting the methods described by Slinkard and Singleton (1977), Chang *et al.* (2002) and Hussain *et al.* (2008) respectively.

Estimation of Total Polyphenols: Gallic acid was used as a standard compound to plot standard curve. Standard stock solution of 1mg/ml concentration of Gallic acid and extracts were prepared in methanol. For standard and extracts, 200 µl were taken from stock solution, then add 200µl FC reagent, 1ml of 15% Na_2CO_3 and make volume to 3ml with methanol. The final mixtures were allowed to stand for 90 minutes and then the absorbance was taken at 760nm. Total phenols are expressed in terms of Gallic acid equivalent (mg/g). Standard calibration curve was used to estimate total phenolic contents (Slinkard and Singleton, 1977).

Estimation of Total Flavonoid Content: Quercetin was used as a standard flavonoid compound and stock solution of standard as well as of extracts was prepared in the concentration of 1mg/ml. Then, 200µl of standard solutions of quercietin and extracts were taken and add 100µl of 10% aluminium nitrate, 100 µl of 1M potassium acetate and 4.6ml of water. After incubation of 45minutes at room temperature the absorbance was noted at 415nm against blank. The concentration of total flavonoid (µg/ml) was determined by calibration curve (Chang *et al*., 2002).

Estimation of Total Polysaccharides: Two hundred milligrams of extract was dissolved in 7ml of 80% hot ethanol, vortexed for 2 minutes, centrifuged at 2700rpm for 10 minutes and supernatant was collected. The procedure was repeated until supernatant gave no color upon anthrone reagent addition and collected residue was dried. Then, it was extracted with 10ml distilled water and 25% HCl (1:1) for 20 minutes at 0ºC and centrifuged at 2700 rpm for 10 minutes. Aliquots (0.1ml) of supernatant were taken and volume was made up to 1ml by adding distilled water. Then, 4ml of anthrone reagent was added, mixed and subsequently heated in thermoregulatory water bath for 10 minutes. Intensity of green color was determined at 600nm against blank. Glucose was used as a standard and standard curve was plotted. All the sample extracts and standards were prepared in triplicates (Hussain *et al*., 2008).

Estimation of Total Glycosaponins: One gram of each extract was dissolved in 50ml of methanol. Then, the contents were refluxed for 30 minutes, by attaching reflux assembly. Then, filter the solution and concentrated to 10ml by using rotary evaporator. The concentrated extract was added drop wise in the tarred beaker containing 50ml acetone and resulting precipitates were dried in an oven at 100ºC. To calculate glycosaponins, weight of precipitates was divided by total weight of extract and multiplied by 100 (Hussain *et al*., 2008).

***In-vitro* Anticancer Activity**

Cell Culture: Human hepatocellular carcinoma (HepG2) cell line was provided by the Centre of Excellence in Molecular Biology (CEMB), PU, Lahore. The cells were maintained in DMEM supplemented with 10% FBS (v/v) and 1% penicillin-streptomycin solution (v/v) at 37ºC, 5% CO_2 and 95% relative humidity.

MTT Assay: The cytotoxic potential of *Onosma hispidum* roots extract was evaluated as described by Mosmann (1983) with slight modifications at University

College of Pharmacy, University of the Punjab, Lahore during the year 2016. Briefly, the cells were plated in a 96-well plate at a density of 2000 cells/ well and incubated for 24 hours, followed by the treatment with different concentrations (50µg/ml, 100µg/ml and 200µg/ml) of extract. After 24 hours, 20µl of MTT reagent (5mg/ml in DMSO) was added in each well and subjected to further incubation for 4 hours, after which 80% of the contents were aspirated from each well. In order to dissolve the formed crystals of purple formazan, 150µl of DMSO was added to each well and kept for 20 minutes at 37°C. The absorbance was then measured using an ELISA plate reader at 500-600nm.

Short Term Proliferation Assay: Short term proliferation assay was performed by the method described by Saeed *et al.* (2012). The cells were seeded in 4-well plates in triplicates at the density of 2000 cells/ well. When70% confluent, media was changed, normal media and plant extract (50µg/ml) were added in control and test wells, respectively. The cells were counted manually at day 1, 3 and 6 using Neubauer chamber to assess the impact of test extracts on cell proliferation.

***In-vitro* Antidiabetic Activity**

Alpha-Amylase Inhibition Assay: The assay was performed using previsouly described method by Kwon *et al.* (2007) with slight modifications at University College of Pharmacy, University of the Punjab, Lahore during the year 2016. Both, the sample (1mg/mL, in methanol) and alpha-amylase (1% w/v) in 1:1 were taken in a test tube and incubated for 5-15 minutes at 37°C. After this, 1ml of potato starch solution (1% w/v) was added followed by incubation for 15 minutes, with subsequent addition of 1ml of 3,5-dinitro salicylic acid color reagent. Mixture was kept in water bath at 85°C for 10 minutes. The mixture was then allowed to cool and absorbance was measured at 540nm against blank using UV-Visible spectrophotometer. One percent (w/v) Acarbose solution alongwith the added reagents served as standard. Percentage inhibition was calculated by using the following formula;

$$\text{Percentage Inhibition} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample})}{\text{Absorbance of Control}} \times 100$$

All the extracts were screened for their antidiabetic potential and the one exhibiting maximum inhibition of enzyme was selected to calculate the median inhibitory concentration.

Non-Enzymatic Glycosylation of Haemoglobin Assay: Modified method of Parker *et al.* (1981) was used for the estimation of non-enzymatic glycosylation of haemoglobin. Briefly, solutions of extracts of roots of *Onosma hispidum* were prepared in methanol and all the reagent solutions were prepared in 0.01M phosphate buffer having pH of 7.4. About 1ml of haemoglobin solution (0.06% w/v), 5µl of gentamycin (0.02% v/v), 1ml of sample solution and 1ml of glucose solution (2% w/v) were taken in a test tube and incubated for 72 hours in a dark environment at 37°C. After 72 hours, the experiment mixture was cooled to room temperature and absorbance was measured at 443nm using UV-Visible spectrophotometer. Tocopherol was used as standard drug in the same concentration as that of extract. Percentage inhibition was determined by using the following formula;

$$\text{Percentage Inhibition} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample})}{\text{Absorbance of Control}} \times 100$$

RESULTS AND DISCUSSION

Extraction and Proximate Analysis: Data regarding the percentage yields of extracts obtained after hot and cold extraction and physicochemical analysis of root powder of *Onosma hispidum* are summarized in Table 1.

Table 1. Percentage yields of Extracts and Physiochemical Properties of Root Powder of *Onosma hispidum.*

Extract	Percentage Yield (w/w)
Petroleum Ether	4.68
Chloroform	5.37
Methanol	7.09
Ethanol	12.06
Aqueous	18.44
Parameter	**Percentage contents ± SD (w/w)**
Moisture content	5.33 ± 0.28
Total ash	12.6 ± 0.17
Acid insoluble ash	5.11 ± 0.01
Water soluble ash	19.96 ± 0.05
Sulphated ash	15.00 ± 0.1
Alcohol soluble extractive	16.73 ± 0.25
Water soluble extractive	19.96 ± 0.05

The result demonstrated that the percentage yield was maximum for aqueous extract while it was minimum for petroleum ether (Table 1). Thus, showing that water has maximum potential to dissolve and extract the constituents of the powdered roots.

Furthermore, various physicochemical parameters like moisture content, ash values and extractive values of the plant material were determined by carrying out preliminary studies (Table 1). The moisture content of the powder was found to be 5.33 ± 0.28 which is within the normal limits and is suggestive of appreciable powder stability (Hussain *et al*., 2009). Moisture content of any crude powder, extract or

formulation plays a significant role in determining its shelf life, efficacy and stability. Presence of moisture in a material poses a substance to chemical and microbial degradation, leading to loss of therapeutic effectiveness (Yu and Anchordoquy, 2009).Similarly, the total ash content was 12.6 ± 0.17 which is an important parameter in assessing the purity and quality of crude powder (Kunle *et al*., 2012). The data suggested high values for water and alcohol soluble extractives, which indicates the presence of sufficient amount of polar constituents like phenols, tannins and flavonoids in the powder (Chanda, 2014).

Phytochemical Screening

Primary Metabolites: The percentage contents of the primary metabolites in the root powder of the plant are summarized in the Table 2.The amount of total lipids, proteins and carbohydrates in the plant is an indicative of its nutritional value. The result of the analysis reveals that the root powder is rich in carbohydrates while the lipid content of the powder is less than the other two metabolites.

Secondary Metabolites: The total content of secondary metabolites in the various extracts of roots of *Onosma hispidum* are listed in Table 2.The result indicates high content of polyphenols, flavonoids and glycosaponins in petroleum ether, aqueous and ethanol extract respectively. Polyphenols and flavonoids are group of related compounds having enormous antioxidant potential. Reactive oxygen species contribute to the majority of the diseases in humans such as cancer, neurodegenerative disorders, cardiovascular disorders and inflammation. Owing to their antioxidant and free redical scavenging effect, polyphenols and flavonoids help in combating these diseases in humans (Conforti *et al*., 2008). In addition, they also play a pivotal role in the management of Type 2 diabetes by enhancing the β-cell function, stimulating the insuline secretion and modulating the enzymes involved in glucose metabolism (Cabrera *et al*., 2006; Iwai *et al*., 2006). Likewise, Laila *et al*., (2014) reported that glycosaponins posses anti cancer, anti diabetic and hepatoprotective potential. Therefore, food stuffs containing these phytoconstituents could be used to prevent different human ailments.

Table 2. Primary and Secondary Metabolites (mg/g) of Root Powder and Extracts of *Onosma hispidum*.

	Primary metabolites		**Percentage contents ± SD (w/w)**	
	Total lipids		4.52 ± 0.02	
	Total proteins		9.48 ± 0.27	
	Total carbohydrates		68.06 ± 0.19	
Extracts	**Total polyphenols**(mg/g)	**Total flavonoids**(mg/g)	**Total polysaccharides**(mg/g)	**Total glycosaponins**(mg/g)
Pet. Ether	107.77 ± 0.57	8.86 ± 0.17	21.67 ± 0.03	53.4 ± 0.45
Chloroform	106.74 ± 1.26	8.67 ± 0.29	26.18 ± 0.05	94.5 ± 0.51
Methanol	105.51 ± 1.26	7.09 ± 0.12	24.53 ± 0.06	1.73 ± 0.25
Ethanol	105.97 ± 1.00	12.26 ± 1.45	36.36 ± 0.61	81.6 ± 0.51
Aqueous	104.21 ± 1.66	47.55 ± 0.13	20.41 ± 0.03	16.5 ± 0.45

***In-vitro* Anticancer Activity:**

MTT and Short Term Proliferation Assay: The results of MTT assay and short term proliferation assay using petroleum ether and aqueous extract of the roots of *Onosma hispidum* are shown in Fig. 1A – D. The findings of MTT assay indicate that extract (petroleum ether) has not shown any significant anticancer effect on HepG2 cell line (Fig. 1A). While using short term proliferation assay, significant results are being shown by the extract on third day of incubation (Fig. 1B). Later on, the growth of cancerous cells again increased. This might be due to the reason that HepG2 cells have developed resistance to the effect of extract on continued exposure. Thus, this anticancer potential of extract may be attributed to its high polyphenolic content as they play a significant role in cancer prevention and control during the initiation, promotion and progression stages (Lambert and Elias, 2010).In addition, various researches involving phytochemicals have shown that plant polyphenols are proven to have hepatoprotective effect due to their ability to protect against oxidative stress (Han *et al*., 2007). The results of MTT assay should be interpreted with care, since incubation time was only 4 hours and any effects due to longer exposures were not determined and might be different from our findings.

The results of MTT assay and short term proliferation assay using aqueous extract of roots of *Onosma hispidum* are shown in Fig. 1C - D. As the results indicate, the extract has not shown any significant anticancer effect on HepG2 cell line using MTT assay. While using short term proliferation assay, significant results are being shown by the extract on third and sixth day of incubation by inhibiting the growth of cancerous cells. These results could be interpreted as, the aqueous extract would show its anticancer potential on HepG2 cell

line on prolonged exposure which might be due to the presence of appreciable amounts of polyphenols and flavonoids. Literature evidences suggest that these phytoconstituents are largely involved in cancer prevention by employing various mechanisms as, induction of apoptosis, prevention of oxidation, stimulation of detoxification enzymes and regulation of the host immune system (Birt *et al*., 2001; Lambert and Elias, 2010).The ability of these constituents in controlling the disease is mainly due to active involvement of their functional hydroxyl groups which mediate their antioxidant effect (Kumar *et al*., 2013).

***In-vitro* Antidiabetic Activity**

Alpha-Amylase Inhibition Assay: The results of *in-vitro* alpha amylase inhibition assay using different extracts of roots of *Onosma hispidum* are shown in the Figure 2A. Among all the extracts obtained after hot and cold extraction, petroleum ether extract has shown the maximum potential of inhibiting the enzyme while the least is being shown by the ethanol extract.

Dibetes mellitus is a complex metabolic disorder with impaired glucose tolerance. In the course of development of newer pharmacological agents, digestive enzymes such as, alpha amylase has served as a potential target. Rhabaso and Chiasson (2004) reported that the inhibition of alpha amylase causes a decrease in the absorption of glucose because it is involved in the breakdown of complex polysaccharides into simple sugars, thereby preventing the elevation of post prandial glucose levels. In the view of available literature, many medicinal plants possessing polyphenolic constituents such as tea leaves, sweet potato and berries exhibited an inhibitory effect on alpha-amylase enzyme (Hara and Honda, 1990; McDougall and Stewart, 2005).

Therefore, maximum inhibition of alpha amylase by the petroleum ether extract of *Onosma hispidum* could be related to its high polyphenolic content. But further investigations are required to elucidate the mechanism of enzyme inhibition and to isolate, purify and characterize the compound responsible for antidiabetic effect.

Non-Enzymatic Glycosylation of Haemoglobin Assay: The results of *in-vitro* assay using different extracts of roots of *Onosma hispidum* are shown in the Figure 2C. Among all the extracts obtained, chloroform extract has shown the maximum activity in inhibiting the glycosylation of haemoglobin while minimal effects were observed for aqueous extract.The antidiabetic effects of *Onosma hispidum* Wall. may be attributed to scavenging of free radicals and possoible regenratrion of pancreatic beta cells (Kumar *et al.,* 2010).

Glucose reacts non-enzymatically with the body proteins to form glucose addition products whose accumulation is considered as a major contribution to long-term complications associated with diabetes (Makita *et al.*, 1991). These glycosylated species are considered as a source of free redicals producing products which aggravate the state of oxidative stress in diabetes mellitus (Adisa *et al.*, 2004). Plant extracts have been found to inhibit the binding of glucose to haemoglobin thereby decreasing the production of free redicals. An increased glycosylation was observed when haemoglobin was incubated with glucose for 72 hrs. However, on addition of plant extract, the glycosylation was siggnificantly reduced. Maximum inhibition of haemoglobin glycosylation was observed by the chloroform extract of *Onosma hispidum* roots which was found to be compareable to that of a standard drug.As data suggested, choloroform extract possessed high polyphenolic and glycosaponin content which is responsible for its antidiabetic potential (Cabrera *et al*., 2006 ;Iwai *et al*., 2006; Laila *et al.*, 2014).Futhermore, various studies have explored that there exist a relationship between type II diabetes and hepatocellular carcinoma. The emergence of HCC is more in patients on insuline and sulphonylureas treatment (Donadon *et al.*, 2009). Therefore, the focus should be shifted to natural sources to prevent such co-morbidities. However, in the present study, further investigations are required in order to identify and isolate the compound responsible for such activity.

Dose-Response Relationship: On the basis of data obtained by preliminary screening, petroleum ether extract having highest enzyme inhibition activity was selected for establishing a dose-response relationship. The results of dose-response relationship are shown in Figure 2B. It indicates that inhibition of alpha amylase is dose dependent with the median inhibitory concentration (IC_{50}) of 80µg/ml. Similarly, as per the results of glycosylation model, chlorofom extract of roots of *Onosma hispidum* showing maximum activity was subjected to further assessment for establishing a dose-response relationship. The results are shown in figure 2D indicating the median inhibitory concentration (IC_{50}) at a dose of 35µg/ml.

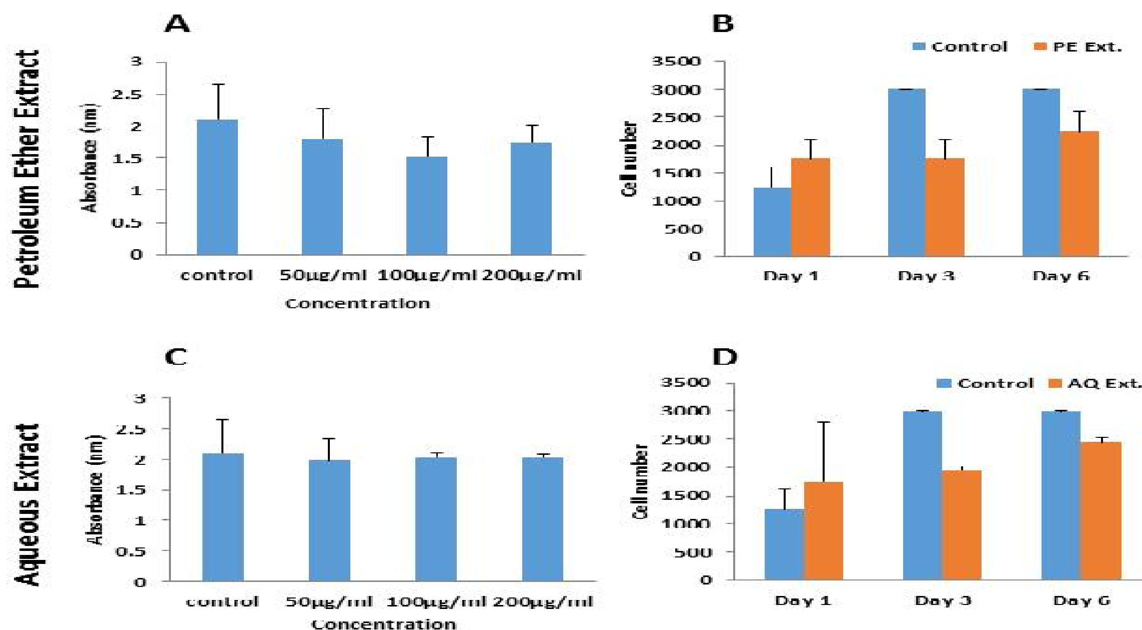

Figure 1. MTT and Short-term Proliferation Assay using Petroleum ether and Aqueous Extracts of roots of *Onosma hispidum*

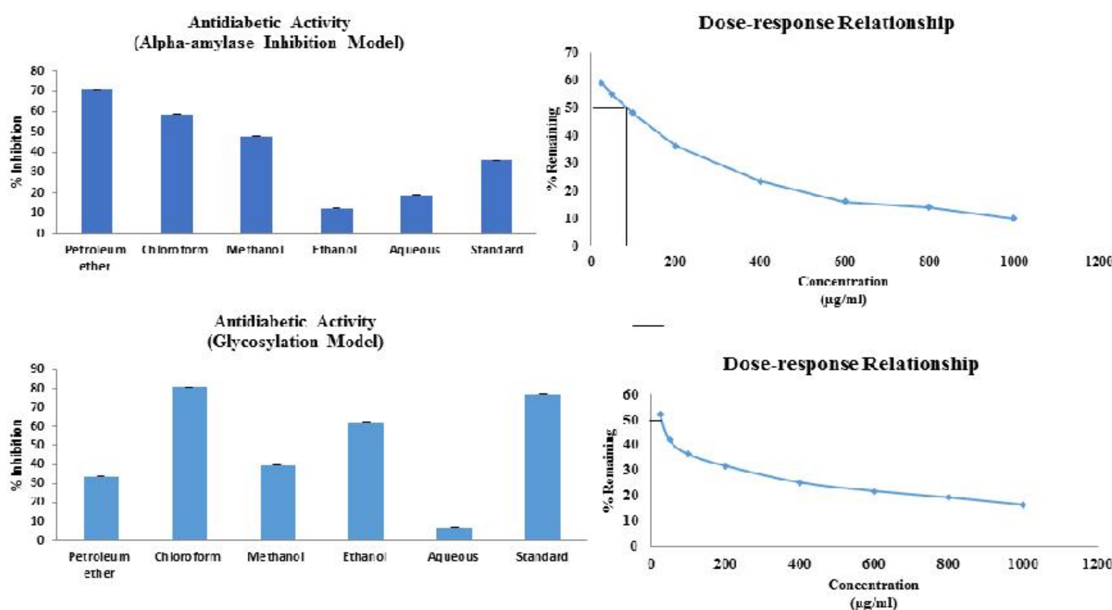

Figure 2. Antidiabetic activity of extracts of roots of *Onosma hispidum. A)* Anti-diabetic activity using alpha-amylase inhibition model, B) Median inhibitory concentration of petroleum ether extract of roots of *Onosma hispidum C)* Antidiabetic activity using glycosylation model D) Median inhibitory concentration of chloroform extract of roots of *Onosma hispidum*

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