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Ultrasonication-assisted extraction, antioxidant activity and α-amylase inhibition potential of *vitexnegundo* leaves

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Original Research Article

ABSTRACT

The current work describes the ultrasonicated assisted extraction followed by comprehensive investigation of antioxidant activity and α-amylase inhibition potential of hydroethanolic leaf extracts of *Vitexnegundo*. Freeze drying assisted ultrasonicated extraction revealed highest level of total phenolic contents (258.29±2.52 mgGAE/g DE) and total flavonoid content (155.91±1.75 mgRE/g DE) in 60% hydroethanolic *V. negundo* leaf extract. Furthermore, total antioxidant power (254.35±1.56 mg AAE/g DE) and antidiabetic effectbased on α-amylase inhibitory assay (IC_{50}=42.91±1.24 µg/mL) was also depicted to be highest in the same extract. Based on current findings, 60% ethanolic extract of *V. negundo*leaveswas revealed to be most potent for its antioxidant and α-amylase inhibition activity, hence could be used for formulation of new plant based functional food sornutraceuticals to improve human health.

Keywords: Functional foods, *Vitexnegundo*, Ultrasound-assisted extraction, Antioxidant activity, α-amylase inhibition

INTRODUCTION

Phenolics and flavonoids are renowned phytochemicals and have gained interest due to their beneficial health effects (Tungmunnithum *et al.,* 2018; Li *et al.,* 2014; Ivanova *et al.,* 2005). These bioactive constituents exist in all parts of the plants and are considered to exhibit imperative role for the management of health risks i.e., oxidative stress, diabetes, coronary heart disorder and cancer etc., (Bazzano *et al.,* 2002; Conte *et al.,* 2016; Kähkönen *et al.,* 1999). Phenolics show their antioxidant effect primarily due to their capability to act as reducing agent, hydrogen donor or to capture/react with free radicals (Debbache-Benaida *et al.,* 2013; Ivanova *et al.,* 2005). Bioactive rich extracts have been revealed to exhibit several biological effects such as antibacterial, antiviral, anti-inflammatory, antifungal, vasodilatory and antiallergic effects (Balasundram *et al.,* 2006; Guo & Xue, 2013;Boudkhili *et al.,* 2015;Dzoyem *et al.,* 2013; Zhang *et al.,* 2016; Ciftci *et al.,* 2015).

The crude extracts obtained from many plants and herbs are considered as valuable source of phenolics and flavonoids, and potentially used for formulation of several functional foods and nutraceuticals (Kahkonen *et al.,* 1999). There is an emerging trend in finding safe natural products for their possible use in functional foods to replace synthetic alternatives having adverse effects and potential toxicity (Boeira *et al.,* 2018; Zhou *et al.,* 2013; Joshi *et al.,* 2012). The marketing trends in this field are quite competitive, so formulation of new types of cheap and quality ingredients is challenging for food processing industries.

Composition of plant extracts depend upon several factors viz., extraction method, plant part selected for extraction, particle size, solvent used for extraction, solvent concentration, temperature and extraction time etc (Tiwari *et al.,* 2011). Phenolics and flavonoids enriched extracts can be achieved by employing different extraction techniques but currently, we have used freeze-drying assisted ultrasonicated extraction method with objective of improved extract yield and to cope with the limitations of conventional extraction methods such as high degree of degradation of bioactive compounds in treated food product, long extraction time and use of excessive solvent etc (Vilkhu *et al.,* 2008).

Vitexnegundo is extensively used aromatic plant in traditional Indian medicinal system. Although all parts of *V.negundo* are employed as medicine but leaves of this plant are considered most effective for medicinal usage. Leaves are shown to exhibit substantial anticonvulsant (Tandon& Gupta, 2005), analgesic (Gupta & Tandon, 2005) and anti-inflammatory (Tiwari &

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Tripathi, 2007) activities, which might be due to high antioxidant potency of its bioactive constituents. Several kinds of polyphenolic compounds including glycosides, iridoids, terpenoids, phenolic, flavonoids and alkaloids are reported as essential constituents of *V. negundo* leaves (Nagarsekar *et al.,* 2011). *V. negundo* can be a promising source of antioxidants (Prakash *et al.,* 2017). Ultrasonicated-assisted extraction (UAE) is an imperative extraction technique since it helps not only in exhaustive or complete extraction of active phyto constituents with less energy and time consumption, but the process ensures greater safety (Boeira *et al.,* 2018). As per literature, the use of ultrasonic waves during extraction ruptures cell wall structure, facilitates the release of its contents and hence increases the extract yield (Abidin *et al.,* 2014; Falleh *et al.,* 2012). So, our goal was to explore the effect of freeze drying assisted ultra-sonication technique on extract yield and evaluation of antioxidant and α-amylase inhibition effect of *V. negundo* leaf extracts *in-vitro*.

MATERIALS AND METHODS

Chemicals

Gallic acid, rutin, acarbose, α-amylase enzyme, Folin-Ciocalteu (FC) reagent were procured from Sigma Aldrich, USA. All other reagents and chemical used were of analytical research grade.

Collection of plant material

Mature fresh *V. negundo*leaves were collected from Azad Jammu & Kashmir, Pakistan. The collected plant was identified and a voucher specimen # UOG-CHEM-20/2018 was deposited at Department of Botany, University of Gujrat, Gujrat, Pakistan.

Sample drying and extraction

Fresh leaves after washing with water and drying with cotton paper, quenched immediately with liquid $N_2$for the preservation of secondary metabolites and freeze dried (Christ Alpha 1-4, LD-German, freeze dryer) at -68°C for 48 hours. The leaves were then ground to fine powder, passed through a 60mesh sieve and stored in Ziplock plastic bag at -80°C until further use. Leaf powder (10 grams) was then subjected to extraction using hydro-ethanolic solvent systems of different compositions (Aqueous, 20%, 40%, 60%, 80% and 100% v/v) at constant temperature of 35±0.2°C for 2 days. The obtained samples were vortexed for 2 hours using mixer (SHO-1D DAIHAN Wise Mix, Scientific Korea) and ultrasonicated (Soniprep-150 ultrasonicator, UK) for 1 hour at 35±0.2°C. Centrifugation was carried out for 10 minutes (13,000 rpm) followed by filtration through filter crucible (containing Whatman grade 42filter paper) connected by a vacuum pump (Todays Rocker-300). After filtration the evaporation of extra solvent was carried out using a vacuum rotary evaporator under same experimental conditions. The resulting crude leaf extracts of *V. negundo* were again freeze-dried at -68°C, extract yield (%) was calculated and stored for future experiments at -80°C.

Determination of total phenolic contents (tpc) and total flavonoid contents (TFC)

TPC present in crude extracts were determined using method as described by Kim *et al,*(2003). Briefly, 0.10 mL of each extract was dissolved in 1 mL FCR (Folin–Ciocalteu reagent) reagent along with the addition of 3 mL of 10% Na_2CO_3 solution(v/v). The mixtures were then heated at 23℃ for 90 minutes. Absorbance was recorded at 750nm by means of a spectrophotometer (Shimadzu UV1700, Japan), gallic acid was used for plotting a standard calibration curve and TFC of each extract was measured in milligrams of gallic acid equivalent per gram dried extract (mg GAE/g DE).

Determination of TFC of each hydro-ethanolic extract was achieved as per previously reported method (Park *et al.,* 2008). Briefly, 0.2 mL of each sample extract was added in a flask containing a mixture of 0.5M $NaNO_2$ (0.10 mL), 30% MeOH (3.4 mL) and 0.15 mL of 0.3 M $AlCl_3.6H_2O$. After keeping the sample mixture for 5 minutes, 1.0 M NaOH (1 mL) solution was mixed and absorbance was noted at 510nm spectrophotometrically. Standard calibration curve was plotted using rutin and findings were recorded as mg of rutin equivalent per gram dried extract (mg RE/g of DE).All experiments were carried out in triplicate.

Total antioxidant power (TAP) assay

The antioxidant activities (*in-vitro*) of different hydroethanolic leaf extracts of *V. negundo* were evaluated based on method reported by (Umamaheswari & Chatterjee, 2008) with minor modification. In short, 0.10 mL of each crude extract was dissolved in reagent solution (28 mM Na_3PO_4+4 mM $(NH_4)_2MoO_4$+0.6 M H_2SO_4). The reaction mixture was heated for 90minutesat 90°Cand cooled to 30°C. Finally, absorbance was

noted at 765 nm utilizing a spectrophotometer (Shimadzu, UV-1700, Japan). The TAP was determined as the number of milligrams of ascorbic acid equivalent per gram dried extract (mg AAE/g DE).

Alpha-amylase inhibition assay

The α-amylase inhibitory effects of understudy hydro-ethanolic leaf extracts of *V. negundo*were assessed according to standard method described by Shai *et al,* (2010) with little modifications. *V. negundo* leaf extracts were mixed with porcine pancreaticα-amylase enzyme (2 units/mL) and 0.10 M phosphate buffer of pH, 6.8. After that, mixtures were heated at 37°C for 20 minutes, followed by the addition of starch solution (1%) and reheated for 1.0 hourat 37°C. The absorbance was recorded at 540nmby a spectrophotometer (Shimadzu, UV1700, Japan) and percent enzyme inhibitory effects were computed using following formula.

$$PI = \left[\frac{\left(\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Sample}}\right)}{\text{Absorbance}_{\text{Control}}}\right] \text{x } 100$$

All the experiments were carried out in triplicate and IC_{50} value of each extract was calculated graphically. Acarbose was taken as reference compound.

Docking study

Docking studies based on Molecular Operating Environment (MOE2016:0802) were also performed to explore the possible inhibitory role of some of the identified metabolites in the understudy hydroethanolic leaf extract with optimal response toward α-amylase inhibition. Docking studies were conducted as per method we have reported previously (Nadeem *et al.,* 2019).

Statistical analysis

All experimental values were subjected to statistical analysis using MINITAB 17.0 software. The analysis of variance (ANOVA) was used to check statistical significance of results and differences were thought/considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Percent extraction yield

Extraction process was optimized by using hydro-ethanolic solvents (having different proportions of water and ethanol) and effect of each solvent composition on percent extract yield was evaluated (Fig., 1). Maximum yield (24.05±0.38%) was obtained by carrying extraction with 60% hydro-ethanolic solvent system (60% ethanol+40% water) whereas minimum yield of 16.33±0.23% was achieved by using 100% water as extracting solvent. ANOVA depicted that extract yield produced by 60% extract was statistically significant from all other extracts ($p<0.05$) while that of 20 and 40% extracts were statistically non-significant ($p>0.05$). Our result regarding % yield of extracts was in agreement with findings of Zhao *et al,* (2014) and Mumtaz *et al,* (2018) who described that during extraction process % yield increases initially, becomes maximum with 60% hydro-ethanolic solvent system and then decreases gradually with increase in concentration of ethanol. Previously, reported extraction yields of ethanolic and petroleum ether *V. negundo* leaf extracts were 15.73% and 1.92%, respectively (Nagarsekar *et al.,* 2010). In another study, the percent yield of chloroform, petroleum ether and aqueous extracts of *V. negundo*root were 2.63%,1.92%, and 2.56%, respectively (Sharma *et al.,* 2018), whereas percent yield of dichloromethane, methanol, ethyl acetate and aqueous extracts of various parts (flower,fruitand leaf) of *V. negundo* was found in the range from 0.53-10.1% (Jeyaseelan *et al.,* 2010). This difference in % yield of the extracts could be due to difference in solvents used for extraction and their composition, extraction method used, storage time, sample particle size, season, plant maturity and geographic distribution (Amessis-Ouchemoukh *et al.,* 2014; Imran *et al.,* 2014).

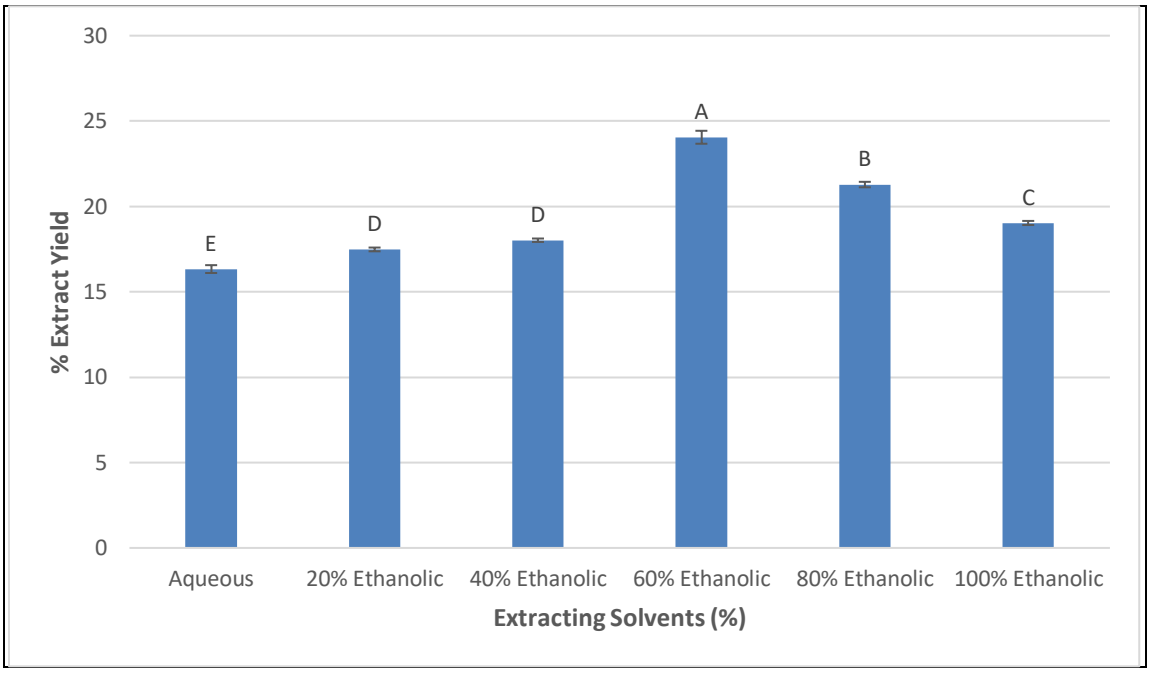

Fig. 1: Percent extract yield of *V. negundo* leaf. Data with different letters (A-E) describe significancedifference (p<0.05)

Total phenolic and total flavonoid contents

Polyphenols are considered as valuable phytochemicals having significant health promoting functions such as anti-hyperglycemic, anti-hypertensive, anticancer and antioxidant effects (Adejoh*et al.,* 2018; González-Sarrías *et al.,* 2013; Yee Lee *et al.,* 2019). Findings related to TPC and TFC of different hydro-ethanolic leaf extracts of *V. negundo*are shown in Table 1.

Highest TPC (258.29±2.52mg GAE/g DE) were observed for 60% ethanolic leaf extract followed by 80%extract (242.30±2.19 mgGAE/g DE), 100% ethanolic leaf extract (199.27±2.41mg GAE/g DE), 40% ethanolic leaf extract (193±2.55 mgGAE/gDE), 20% ethanolic leaf extract (171.44±2.87mg GAE/g DE) and aqueous extract (143.23±2.73 mgGAE/gDE). Likewise, maximum TFC (155.91±1.75 mg RE/g DE) were found in 60% ethanolic leaf extract followed by 80% ethanolic extract (145.34±2.84 mg RE/g DE), 100% ethanolic leaf extract (131.3±1.80mg RE/g DE), 40% ethanolic leaf extract (123.59±1.93mg RE/g DE), 20% ethanolic leaf extract (104.19±1.77mg RE/g DE) and aqueous extract (87.24±1.89mg RE/g DE).

Table I: Tpc and Tfc of *v. negundo* leaf extracts

Extracts	TPC (mg GAE/g DE)	TFC (mg RE/g DE)
Aqueous	143.23 ± 2.73^{E}	87.24 ± 1.89^{F}
20% ethanol	171.44 ± 2.87^{D}	104.19 ± 1.77^{E}
40% ethanol	193.0 ± 2.55^{C}	123.59 ± 1.93^{D}
60% ethanol	258.29 ± 2.52^{A}	155.91 ± 1.75^{A}
80% ethanol	242.30 ± 2.19^{B}	145.34 ± 2.84^{B}
100% ethanol	199.27 ± 2.41^{C}	131.3 ± 1.80^{C}

Each value in the table is represented as mean ±standard deviation (n=3). Means with different letters as superscript $^{(A-E)}$ in the columns are significantly (P<0.05) different from each other

The difference in the amounts of TPC and TFC could be ascribed to distinct polarity or solubility of phenolic and flavonoids in different solvent systems. Moreover, it has also been mentioned in literature that solubility of polyphenolic compounds depends upon degree of polymerization, formation of insoluble complexes and interactions of the phenolic with other food constituents (Falleh *et al.,* 2008; Sánchez-Mundo *et al.,* 2016). In several studies, researches have reported a good amount of phenolics and flavonoids in this plant (Dar *et al.,* 2017; Fatimatuz Zahura Falguni, 2017; Janakiraman & Jeyaprakash, 2015; Kumar *et al.,* 2010; Lakshmanashetty *et al.,* 2010).

Total antioxidant power (tap) of *v. negundo*leaf extracts

The phosphomolybdenum assay is one of the frequently used methods for quantitative measurement of TAP of understudy extracts. During reduction of Mo (VI) into Mo (V), a green colored phosphomolybdenum (V) complex is obtained in presence of tested extracts with absorbance maximum at 695 nm. Antioxidant capability shown by 60% ethanolic leaf extract of *V. negundo* i.e., 254.35±1.56 mg AAE/g DE was higher than 80% ethanolic leaf extract (244.00±3.76 mg AAE/g DE), 40% ethanolic leaf extract (25.01±235 mg AAE/g DE), 100%ethanolic leaf extract (219.59±1.88 mg AAE/g DE), 20% ethanolic leaf extract (190.98±1.86mg AAE/g DE) and aqueous leaf extract (173.93±3.88 mg AAE/g DE) (Fig., 2).

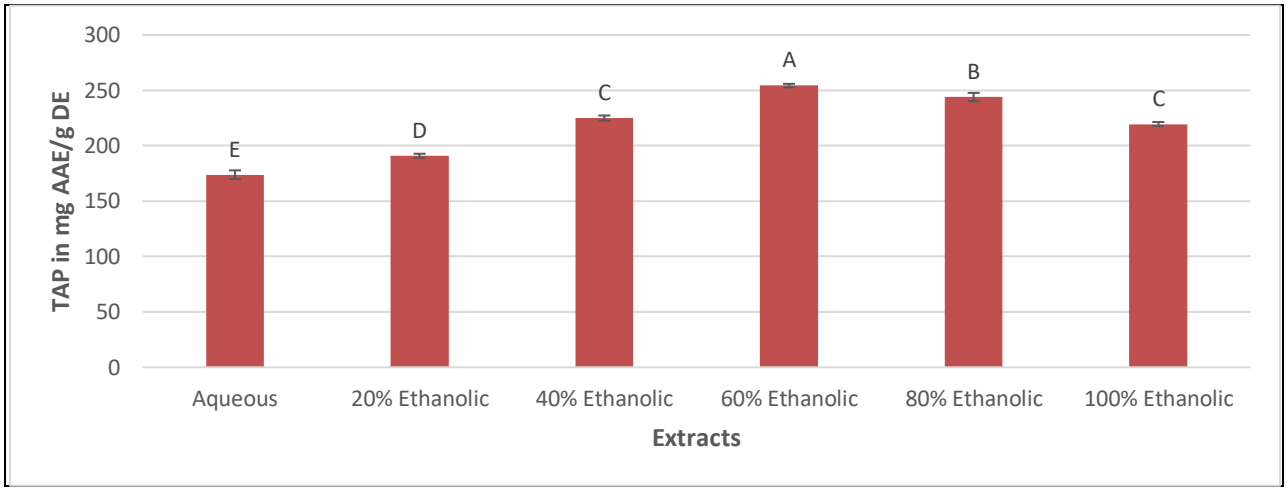

Fig. 2: TAP of *V.negundo* leaf extracts.Superscripts (A to E) indicate significance difference

The ANOVA clearly indicated that, the difference of mean between 60% and other extracts was significant statistically (p<0.05). However, TAP was non-significant statistically (p>0.05) in case of 40% and 100% hydro-ethanolic leaf extracts of *V. negundo*. In a previous work, TAP vales for ethanolic and methanolic leaf extracts of *V. negundo*has been reported to be 214.81±4.07 mg AAE/g DE and 23.21 mg/100 of AAE/g DE, respectively (Kumar *et al.,* 2010; Lakshmanashetty *et al.,* 2010). The variation in antioxidant activity might be due to different extraction technique and solvent used for extraction. Hence, 60% extract was established as the most potent among all other understudy extracts with highest antioxidant potential.

Alpha-amylase InhibitoryActivity

Rapid increase in rate of type-2 diabetes mellitus is one of the major health issues worldwide (DeVille-Almond *et al.,* 2011). In type-2diabetes, hyperglycemia is accompanied with an aberrant escalation in postprandial blood glucose level. Many herbal extracts are investigated till date to suppress production of glucose from carbohydrates in gut and its absorption within intestine (Matsui *et al.,* 2007). The α-amylase catalyzes the hydrolysis of oligosaccharides, glycogen and starch to simplE sugars which are then readily absorbed in intestine. So, inhibition of theenzyme ‘α-amylase’is believed to be valuable in controlling diabetes by reducing intestinal absorption of glucose (Gautam *et al.,* 2013).

All understudy extracts of *V. negundo* leaves showed considurable *in-vitro* α-amylase inhibition activities (Fig., 3).

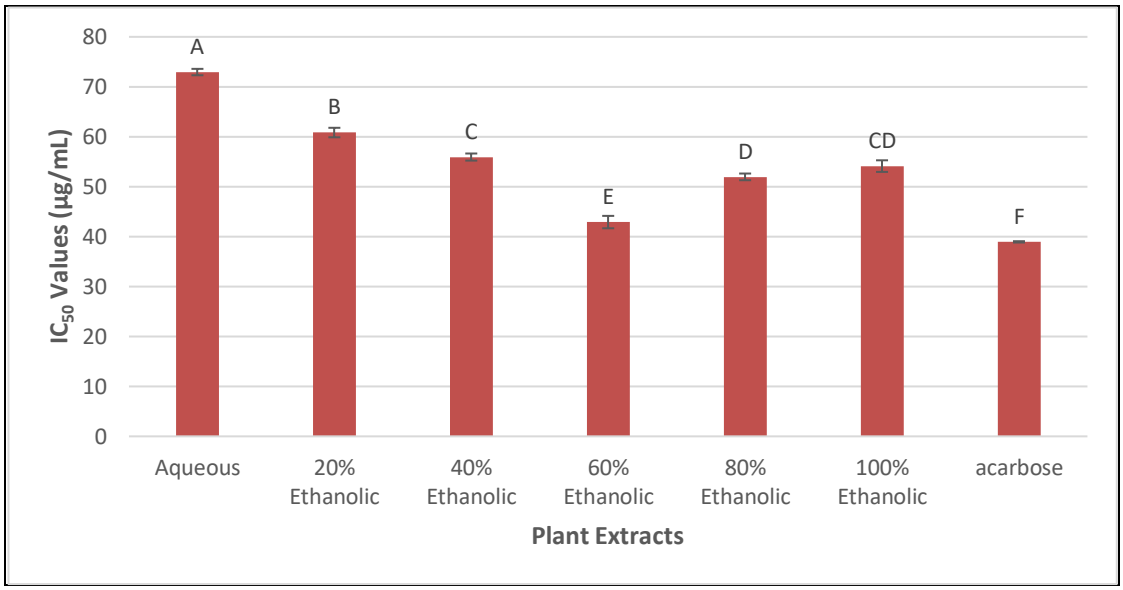

Fig. 3: The α-amylase inhibitioneffect of hydroethanolic extracts of different concentrations.Data was examined by one-way ANOVA, letters as superscript indicate significance difference among values (p<0.05)

The α-amylase inhibition effect of 60% hydroethanolic leaf extract was the most potent with IC_{50} value of 42.91±1.24 µg/mL (p<0.05) and was compareable to standard α-amylase inhibitor i.e. acarbose (IC_{50}=38.95±0.13 µg/mL). The inhibition of α-amylase byaqueous extract was minimum among all tested extracts with IC_{50}, 72.95±0.64µg/mL. IC_{50} values of 40, 80 and 100% hydro-ethanol leaf extracts regarding α-amylae inhibition were 55.93±0.71 µg/mL, 51.96±0.167 µg/mL and 54.12±1.16 µg/mL,respectively which were statistically non-significant (p>0.05). Previously reported IC_{50} values for flower, root, stem and leaf extracts of *V. negundo*regarding α-amylase inhibition effect are 0.5, 0.8, 0.08 and 1445.43 mg/mL, respectively (Gautam *et al.,* 2013). In anotherinvestigation, α-amylase inhibition effect of *V. negundo* leaf extract is reported to be 0.21±0.014 mg of maltose (Devani *et al.,* 2013). The difference in enzyme inhibition potential of extracts could be attributed by different amounts of extracted phenolic and flavonoid contents, which in tern might be due to diference in exration method and solvent used for extraction.

To explore the possible inhibitory role of some of the identified metabolites in the understudy hydroethanolic leaf extract (with optimal response regarding α-amylase inhibition) docking simulations towards α-amylase were performed using MOE. For pose-study and binding orientation, we selected compounds i.e., Luteolin 7 glucoside, negundoside, *p*-hydroxybenzoic acid and protocatechuic acid having binding affinity values -7.4320, -7.6506, -6.6314 and -4.1354 kcal/mol, respectively. The binding cleft of α-amylase lies deep near its center and consists of Asp197, Glu233 and Asp300. While, the active site consists of several aromatic residues and side chains. Aromatic residue present are: Ala307, His305, His299, Tyr258, Ile235, Pro163, His101, Tyr62, Trp59 and Trp58. The side chains of Asp236, Lys200, Asp165 and Arg61 are also important. It is observed from the 3-D interaction plot presented in Fig.,4 (**a-d**) that these compounds interact with above mentioned key residues of binding sites.

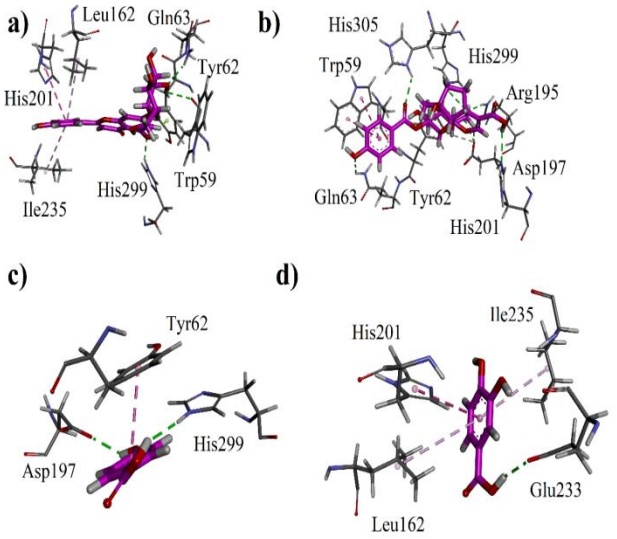

Fig. 4: (**a-d**) 3-D interaction plot of identified phytochemicals (9-12), respectively in active sites of porcine pancreatic α-amylase

CONCLUSION

Conclusively, the 60% hydro-ethanol extract of *V. negundo*leaf appearedto be the most effective extract in respect to TPC, TFC, TAP and α-amylase inhibition potential among tested extracts.Present work has shown that *V. negundo* leaf extracts exhibit potent α-amylase inhibition activity hence might be used for futuristic development of anti-diabetic functional foods or phyto-pharmaceuticals.

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