Volume 1, Issue 2. August, 2021. www.jbbt.org

FERMENTATION OF WHEAT STRAW FOR THE EXTRACTION ANDCHARACTERIZATION OF INDUSTRIALLY IMPORTANT ENDOGLUCONASE BY *PHAEOLUSSPADICEUS*

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ABSTRACT

Pakistan is an agricultural country but the burning of agricultural waste presents a major threat to the environment and human beings by producing smog. On the other hand, industrial enzymes are widely used in many industries including food, chemical, medicine and drug *etc*. But the high cost of industrial enzymes limits their uses. In this study, microbial source of enzymes was used to degrade substrate and the agricultural waste was utilized as a substrate for fermentation process. Agricultural waste, also known as lignocellulosic biomass, is widely available and hence, it provides a cheap substrate for industries. Endoglucanase enzyme was produced and extracted from *Phaeolusspadiceus* using wheat straw as the substrate. The study reported that maximum activity of endoglucanase was observed after 96 hours (4 days), at 25-30˚C, at 50% moisture level and pH 4.5. Moreover, addition of carbon source (5% sucrose) and nitrogen source (2.5% urea) enhances the production of endoglucanase. After description to find out its optimum temperature, pH, effect of different metal ions and Km and Vmax. Endoglucanase activity at different conditions presented that the optimum temperature was 30˚C and pH was 4.5. And values of Km and Vmax showed that endoglucanase has high empathy for its substrate.

Keywords: Endoglucanase, Enzyme Kinetics, Fermentation, *Phaeolusspadiceus*

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Introduction

Pakistan is an agricultural country and one of the major producers of important crops like wheat, maize, rice, cotton *etc*. Agriculture serves as the backbone of the country's economy. However, as a result of cutting crops, a huge amount of agricultural waste is produced(Rabbani and Fatmi, 2018). This agricultural waste is called *lignocellulosic biomass*. Due to the unawareness among farmers, they burn this agricultural waste. Burning of agricultural waste may lead to the environmental pollution such as production of smog. Smog leads to many health problems related to respiratory system and also leads to depletion of ozone layer(Katongole, 2009;Azam *et al*., 2015).

On the other hand, for the agriculture sector, there is an increased demand of industrial enzymes for the production of fertilizers, insecticides, pesticides etc. As Pakistan's economy relies mainly on agriculture, the country needs to import industrial enzymes for agricultural industries(Gurung*et al*., 2013). Besides agriculture, industrial enzymes have also many applications in other sectors including food, medicine and drugs, textile, cosmetics *etc*. Industrial enzymes provide many benefits such as high specificity and sensitivity, reusability and biodegradability. But at the same time, they are expensive to produce (Robinson, 2015).

From the above discussion, we come to know about two problems:

1. Environmental pollution due to the burning of agricultural waste
2. High cost of industrial enzymes

As enzymes can be extracted from the cells of plants, animals and microbes, we had used the microbial source of enzymes. Microbial enzymes are preferred to use in industries on large scale as the microbes are easy to produce. Microbes can easily be produced on large scale in the form of large colonies and thus, a large amount of amount of enzymes can be extracted from

their cells. Microbial enzymes are easy to use and handle and they provide the reasonable, biodegradable and cheap source of enzymes. Bacteria and fungi are mainly used for the production of microbial enzymes (Lynd *et al*., 2002).

Furthermore, the agricultural waste, also known aslignocellulosic biomass, serves as the cheap and easily available substrate in industries. It is the rich source of carbon and has high percentage of cellulose in its composition.It is being used as the substrate for many industrial processes including solid state fermentation (Ahmed *et al*., 2009). Microbes can use this lignocellulosic biomass as a substrate to convert it into simple sugars by the action of cellulytic enzymes (Singh *et al.,* 2016). Examples of lignocellulosic biomass are wheat straw, sugarcane bagasse *etc*.

In our research work, solid state fermentation (SSF) was conducted in which *Phaeolusspadiceus* was used for the extraction of endoglucanase and wheat straw was used as substrate. *P. spadiceus* degraded wheat straw by the action of extracellular enzymes including endoglucanase and exoglucanase. So, these extracellular enzymes could easily be extracted from the medium after centrifugation.

MATERIALS AND METHODS

Substrate

Wheat straw was used as a substrate for the expansion of*Phaeolusspadiceus* to provide endoglucanase.Wheat straw was elected because it's low-cost, contain high share of polysaccharide (30-40%), simply accessible in West Pakistan and sensible for the expansion of cellulolytic microorganisms (Ahmed *et al.*, 2009).

Substrate Collection

Wheat straw was collected from the rural area of Mirpur (AJK) and then air dried for 10 days. It was then ground to powder and packed in air tight jars for successive use in fermentation method.

Fermentative Organism

The collection of *P.spadiceus* was obtained from Sozo Adventure Park near lower topa, Murree, used for the assembly of Endoglucanase. First take the loop packed with spores of P. spadiceus and then shifted on Malt Extract Agar media (pH=5.0) aseptically. Place the media plates in incubator for 3-4 days for growth. After incubation, store the plates at 4^{o} C for further use.

Inoculum Preparation

From the culture plates of *P. spadiceus*, a portion of species growth is inoculated aseptically to the Malt Extract broth media (pH=5.0) in flask. Place the flask in shaking incubator at 30^{o} C and 180 rpm for 2-3 days.

Fermentation Process

Solid state fermentation (SSF) method was used for the assembly of Endoglucanse by *P.spadiceus* victimization wheat straw as a carbon supply. Flasks having 5g of powdered wheat straw were moisten with 2.5ml (30%) of distilled water having pH 5.5 (maintained with the assistance of 1M HCL and 1M NaOH). Every flask once autoclaved was inoculated aseptically with 1ml of *P. spadiceus*. These flasks then incubate at 30℃ for specific day (Mahmood *et al*., 2013).

2.6 Sample Harvesting

After specific days, contact technique was used for the extraction of endoglucanase (Krishna *et al*., 1996). We add 50ml distilled water in flasks. These flasks were then shake at 120rpm for 30 minutes in shaking setup. After shaking, the mixture was filtered. Filtered catalyst extract was then centrifuged at 6000 rpm for 10 minutes. Supernatant was stored as a crude enzyme extract at 4℃for enzyme assay (Shafique*et al*., 2004).

Effect of Conditions on Enzyme Production

Enzyme production was optimized at different conditions to observe the maximum enzyme production by *P. spadiceus*.

Effect of fermentation period*:* The fungus was grown for the specific days *i.e.* 24 hours to 168 hours to check maximum enzyme production.

Effect of pH: Five different pH i.e. 3.3, 4.5, 5.5, 6.5, 7.5, 8.5 of distilled water were used for giving moisture (50%).

Effect of temperature and moisture:In order to visualize impact of temperature and moisture different types of temperature has been adjusted (25^{o} C, 30^{o} C, 35^{o} C and 40^{o} C) and similarly, different moisture level 30 %(1.5ml), 50 %(2.5ml), 70 %(3.5ml) have been utilized.

Effect of Carbon and Nitrogen sources: Three different Carbon sources i.e. Glucose, Fructose and Sucrose and three different Nitrogen sources i.e.Peptone, Urea and Ammonium nitrate were used in combination to observe the maximum enzyme production.

Enzyme Assay

*Standard:*Glucose (molecular weight =180g/mole) was used as a standard. Different concentrations of standard were prepared and the absorbance of each concentration was measured at 540nm.

*Substrate:*Carboxy methyl cellulose (CMC) was used as the substrate of Endoglucanse. 1% of CMC in 100mL of distilled water was used for endoglucanase essay.

Reagents used:

Dintrosalicylic Acid (DNSA) was used for colorimetric testing with the hydrolytic products of endoglucanase. 500 ml solution of DNSA was prepared.

Sodium citrate buffer was used to control the pH of assay. Sodium citrate buffer having the pH of4.8-5.0 was utilized. The pH was monitored with the help of pH meter and was adjusted with the help of NaOH and HCl.

Principle of enzyme assay*:* DNSA react with the hydrolyzed product of endoglucanase and formed colored complexes. The absorbance of these complexes was then measured by spectrophotometer at 540nm. Their concentrations were determined by comparing their absorbances with the standard. Increase in the concentration of enzyme also increase in the concentration of hydrolyzed product. More the hydrolyzed product more the colored complexes and hence absorbance of sample will be increase (Iram *et al*., 2021)

Procedure of Enzyme Assay*:* To check the activity of endoglucanase,1ml of crude enzyme was added into 1ml of 1% of substrate (CMC) in test tube. Sodium citrate buffer having the pH 5.0 was added to maintain pH (Shafique *et al*., 2004). Then the test tube was incubated at 27-28 for 30 minutes in the incubator. After incubation of 30 minutes, 3ml of DNSA was added in each test tube and were incubated in boiling water for15 minutes. In boiling water, DNSA reacted with the digested products and formed colored complexes. The absorbance of these complexes was then measured by spectrophotometer at 540nm.

Enzyme Activity*:* One unit of enzyme activity is defined as the amount of enzyme which released one micromole of glucose per minute.

Characterization of Endoglucanase

Endoglucanase was exposed to characterization of different kinetics parameters.

Effect of temperature*:* Optimum temperature was characterized for the maximum activity of endoglucanase. This catalyst assay was performed at different temperature (20℃, 25℃, 28℃, 30℃, 35℃, and 40℃).

Effect of pH*:* Enzyme assay was performed with buffer solutions of different pH. Sodium citrate buffer of different pH*i.e.*3.5, 4.5, 7.5 and phosphate buffer *i.e.*6.5, 7.5 were used.

Effect of metals ions*:* The enzyme activity is dependent upon its structure. Metal ions alter their structure once they interact with protein either inhibit or enhance their activity. Endoglucanase assay was then performed by adding 5 completely different metal ions ($CaCl_2$, KCl, NaCl, and $MnCl_2$).

Effect of concentration of substrate on Endoglucanase for the production of Km and Vmax: Endoglucanase was additionally characterized by studying the impact of concentration of enzyme substrate on enzyme activity. Five different concentrations of CMC *i.e.* 2 mM, 4mM, 6mM, 8mM, and 10mMwere used and assay was performed in every concentration.

RESULTS AND DISCUSSIONS

Endoglucanse is the most essential enzyme which is used to reduce large amount of lignocellulotic waste. Effect of different conditions and manufacture of endoglucanase from the

fungus *P. spadiceus* will help to produce more and more enzyme, decrease pollution and environmental issues and produce advantageous things from resulted fermentable sugar. The different factors were adjusted during study would discuss currently.

Optimization of Conditions

Effect of fermentation period*:* The result achieved from the fermentation period has been shown in table 3.1. The activity of endoglucanaseis increased by increasing time period and rises to maximum before 72 hours (Maximum activity= 21.05 IU/mL/min) then show small reduction up to 96 hours.

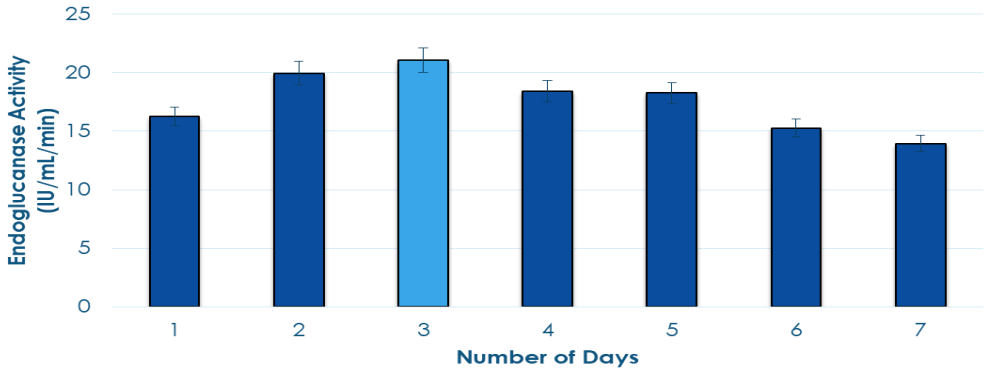

Figure 1. Activity of Endoglucanase produced from *P. spadiceus* under varying fermentation period

Effect of pH*:* Solid state fermentation was optimized to produce abundant endoglucanase from *P. spadiceus*. Change in the pH affects the ionic strength of growth media. Fungus was grown and give the maximum activity at pH 4.5 (Maximum activity = 32.28 IU/mL/min). Increase in pH of the growth media decrease the production of Endoglucanase in fig 3.2.

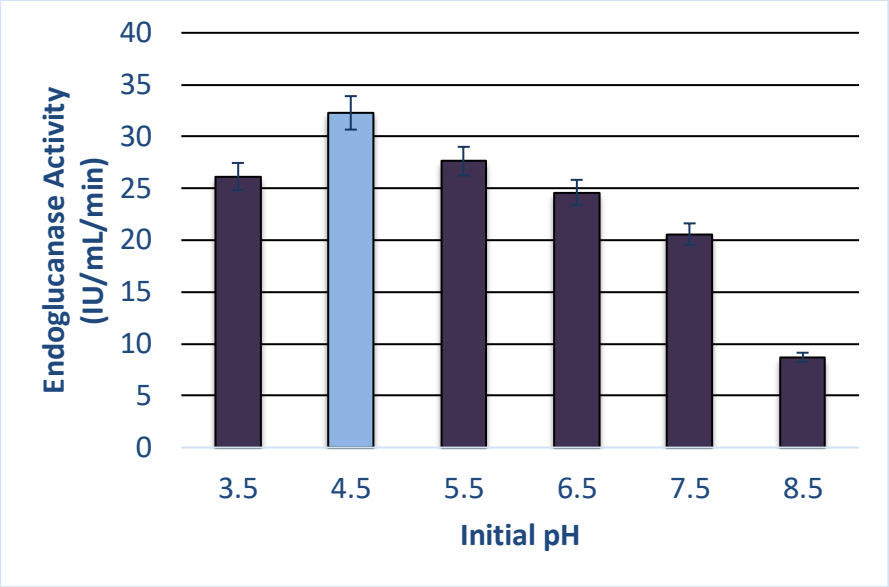

Figure 2. Activity of Endoglucanase produced by *P. spadiceus* under varying pH

Effect of temperature and moisture: Temperature and moisture play an important rolein the growth of *P.spadiceus*. During the optimization of temperature and moisture, it has been observed in my research that maximum endoglucanase was produced at 30℃ and 70% moisture level having activity 18.71 IU/mL/min**.** This indicate that the fungus has maximum growth rate at 30℃ and 70% moisture level (fig 3.3).

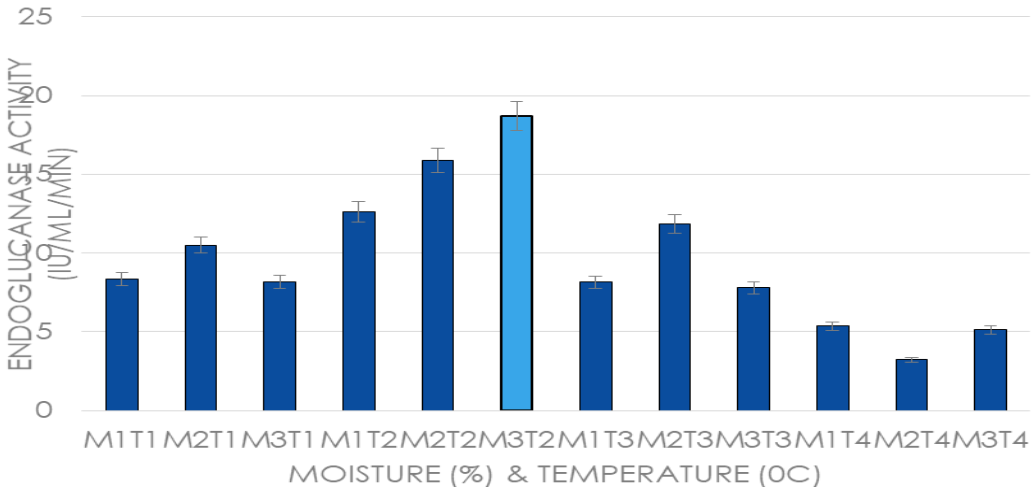

Figure 3. Activity of Endoglucanase produced by *P. spadiceus* under varying temperature and moisture

Effect of Carbon and Nitrogen sources*:* For the improvement of fungus growth, direct Carbon and Nitrogen sources are provided. Maximum enzyme activity was observed in the presence of glucose and urea.

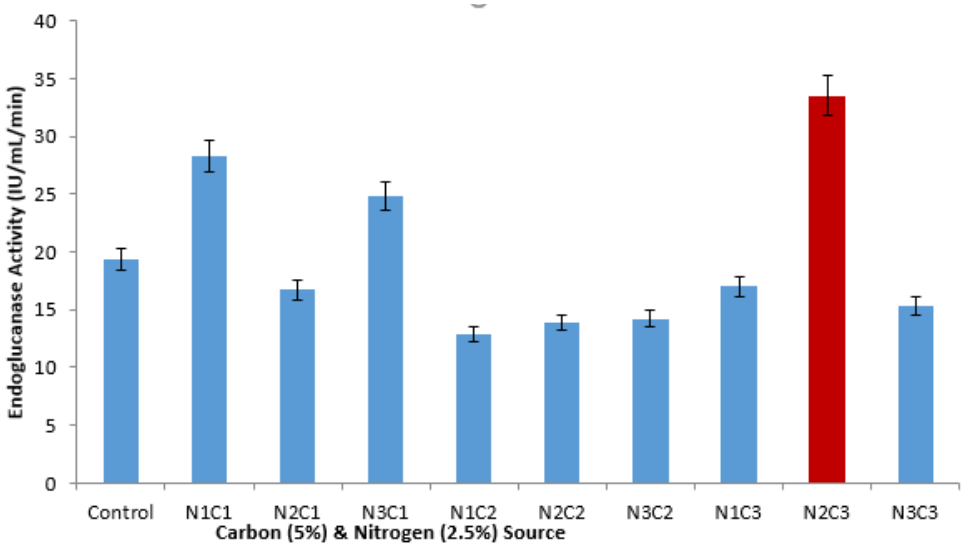

Figure 4. Effect of different Carbon and Nitrogen sources on endoglucanase activity

Characterization of Endoglucanase

Determination of optimum pH*:* Change in pH would affect the ionic strength of the growth

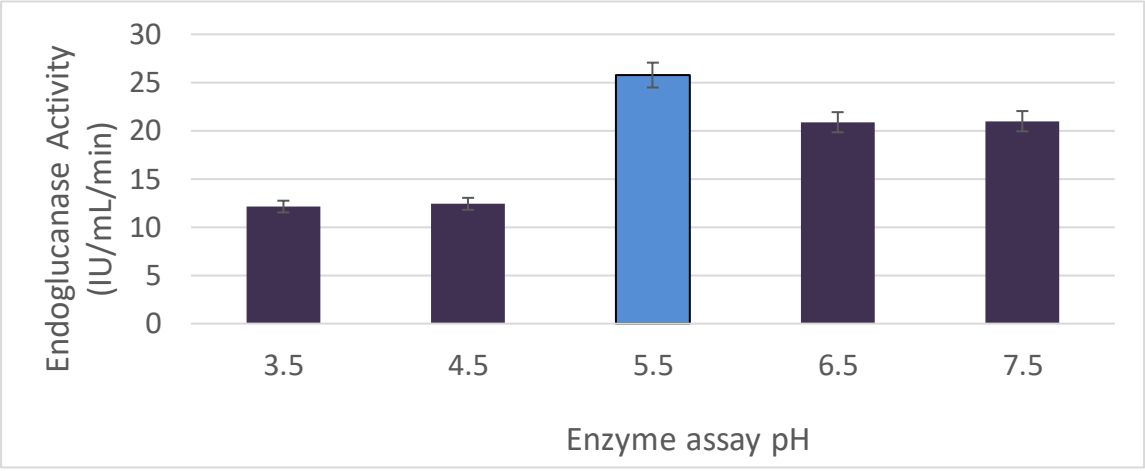

Figure 5. Activity of Endoglucanase produced by *P. spadiceus* under varying optimum pH

media. Fungus was grown at five different pH of buffer solution but maximum activity was found at pH 5.5 (Maximum activity = 25.77 IU/ml/min). Increase in the adjusted pH of buffer solution increase in the production of endoglucanase in fig 3.5.

Determination of optimum temperature*:* During the determination of optimum temperature, it has been observed that maximum endoglucanase was produced at 28℃ having the activity 27.08 IU/mL/min shown in fig 3.6.Further increase in temperature decrease the production of endoglucanase because at a very high temperature enzyme is denatured.

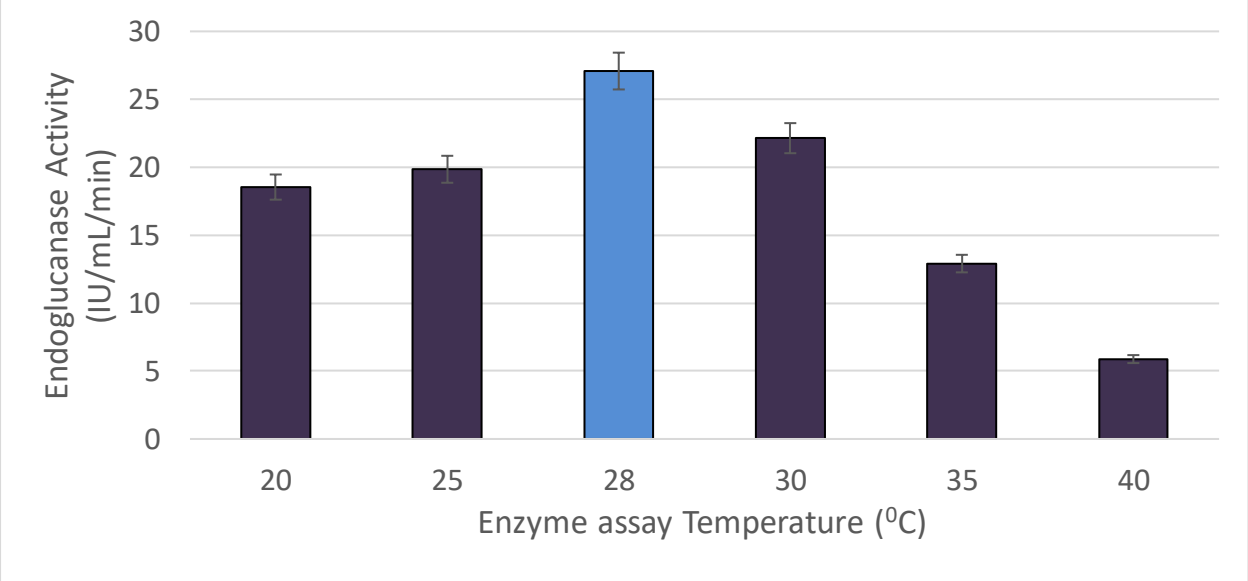

Figure 6. Activity of Endoglucanase produced by *P. spadiceus* under varying optimum temperature

Effect of metals ions*:* results showed that some metals ions have positive effect and some have negative effect on the production of endoglucanase, because those metal which act as cofactor have increase production of endoglucanse and those which act as inhibitor decrease endoglucanase production shown in fig 3.7.

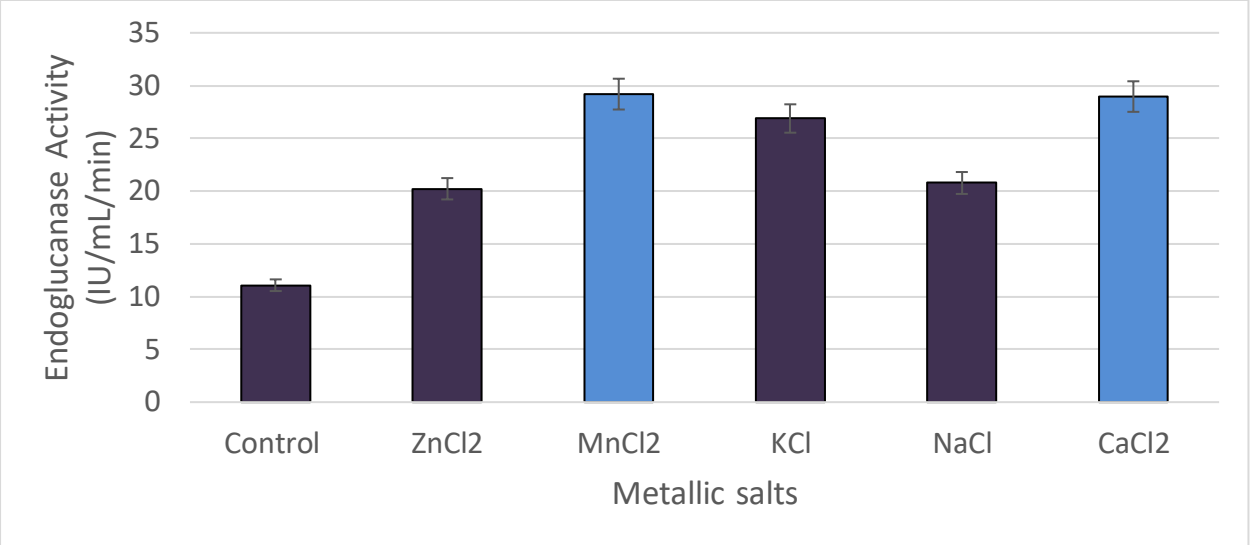

Figure 7. Activity of Endoglucanase produced by *P. spadiceus* under varying Metals Ions

Effect of substrate concentration on Endoglucanase*:* Results showed that increase in concentration of substrate caused an increase in the velocity of enzyme upto a certain limit. After that the rate became constant and further increase in substrate concentration had no effect (fig 3.8). The reason was that all the active sites were occupied with substrate. Line weaver Burk plot the graph between inverse of maximum velocity and inverse of Km respectively**.**

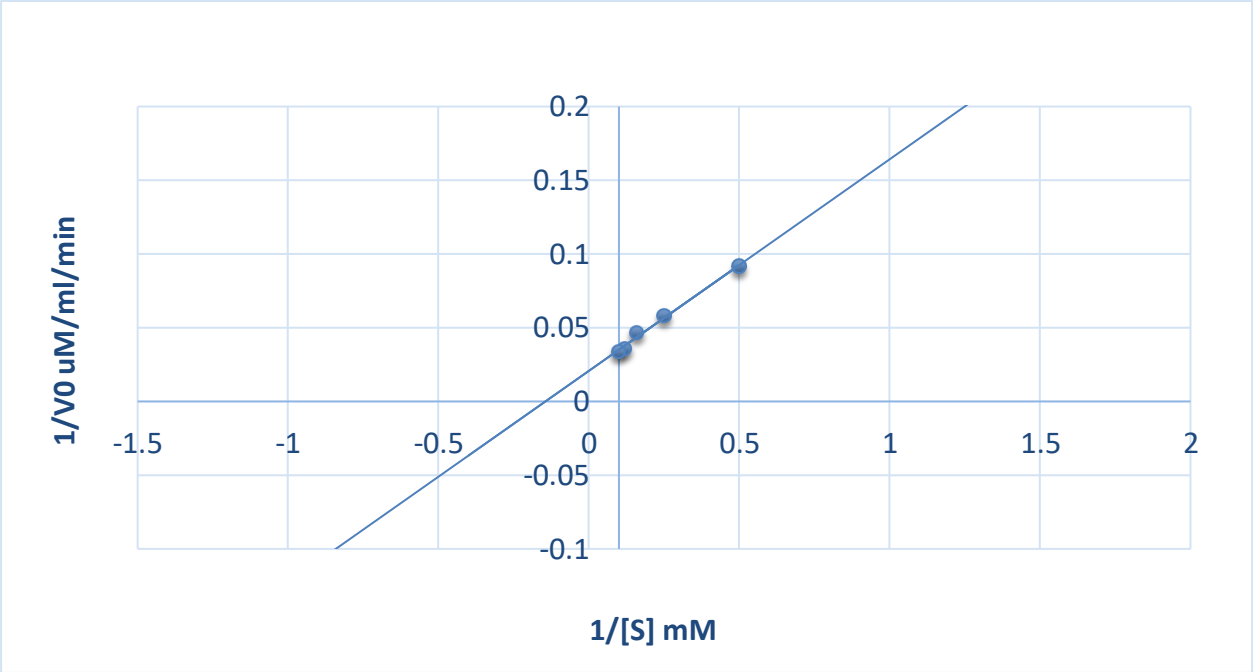

Figure 8. Line-Weaver Burk double reciprocal Plot to calculate K_m and V_{max}

CONCLUSIONS

The current study was approved out to harvest and improve endoglucanase by *P.spadiceus*. The lignocellulosic biomass in the form of wheat straw was used as a substrate for fermentation process. To find extreme yield of endoglucanase, effect of different parameterswas observed including, fermentation period, temperature, pH, moisture level and different carbon and nitrogen bases. Crude enzyme extract obtained after centrifugation was then characterized for temperature, pH, metal ions and substrate concentration for the purpose of Km and Vmax. Maximum activity of endoglucanase was observed after day 4, at 25-30˚C, at 50% moisture level at pH 4.5. Moreover, addition of carbon source (5% sucrose) and nitrogen source (2.5% urea) effect of manufacture of Endoglucanase. After description to find out its optimum temperature, pH, effect of different metal ions and Km and Vmax. Endoglucanase activity at different conditions presented that the optimum temperature was 30˚C and pH 4.5 and values of Km and Vmax showed that endoglucanase has high empathy for its substrate.

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Volume 1, Issue 2. August, 2021. www.jbbt.org

CHEMICAL ANALYSIS AND BIOACTIVITIES ASSESSMENT OF *PINUS* NEEDLES LEAVES

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ABSTRACT

Pinus needles leaves have long history of its uses as food medicines and cosmetics in different parts of world . In current study *Pinus* needles leaves of different trees were evaluated for their proximate, total phenols, total flavonoids, tannins and total oil contents by using different methods as well as GC-MS and FT-IR techniques . Whereas various antioxidants, antibacterial ,anti mycobacterium tuberculosis and cytotoxicity assays were performed to determined bioactivities of different extracts of *pinus* needles leaves. Results indicates that pinusRoxburgiihave provided higher levels of moisture (18.5 ± 2.6 %), ash (2.4 ± 0.5 %) , dietary fiber (24.±2.8 %), crude lipid (8.5 ± 0.7 %), Carbohydrates (45.5 ± 2.5 %), total phenols (7.26±1.52) flavonoids (14.53±2.45), tannins (4.36±1.23) and total oils (1.92 ± 0.28) contents as compared to other extracts analyzed. GC-MS analysis exposed higher quantity of some saturated essential fatty acids in *Pinus*needles leaves, those were further confirmed by indication of their functional group by FT-IR analysis. According to results methanolic extracts of Pinusroxburgii has higher provided higher antioxidant values (DPPH, IC_{50} = 38.36±4.58 µg/ml) and higher zones of inhibition for various bacterial and anti mycobacterium strains. Whereas lower values of brine shrimp cytotoxicity assay depending on concentration of extracts was obtained for Pinusroxburgii as compared to other extracts analyzed. These results indicates that pinus needles leaves especially Pinusrox burgii has greater potential of antioxidants , antibacterial, anti mycobacterium activities as well as reliable cyto toxicity due to phenolic, flavonoids and essential oils presents in extracts.

Key words; *Pinus* needles leaves, Chemical anlaysis, Organic acids, Bioactivities

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Rawalpindi_____

INTRODUCTION

A therapeutic plant has same properties as regular pharmaceutical medications. People have utilized them all through history to either cure or decrease side effects from an ailment. A pharmaceutical medication is a medication that is created in a research center to cure or help a disease. Names of some of the medicinal plants are: Amla, Ashok, Bael, BhumiAmla, Brahmi, Chiraita, Gudmar, long peeper *etc* The plant is utilized as a superior tonic in treatment of fever and for curing different skin infections (Alanis *et al.*, 2003; Maryam *et al.*, 2021).

Around 80% of world population depends on home grown solutions for treatment of different human afflictions. Therapeutic plants have the fundamental part in allopathic medications, extensive number of current medications, for example, dioxin, morphine, codeine, ibuprofen, vinblastine, cocaine, emetine, ephedrine, vinocristine, pilocarpine and so forth., have been disengaged from plants (Ahmad *et al.*, 2014). After greater progression of manufactured science during the later half of twentieth century over common items, again the enthusiasm of pharmaceutical industry in restorative plants stir and they took a gander at wellspring of natural plants as synthetic platforms for amalgamation of medications. The normal compounds from therapeutic plants are more secure and could be found to supplant the engineered drugs which constitutes around 70% of our medications (Al-Snafi, 2013)..Numerous medicinal plants contains awesome amount of antioxidant compounds instead of vitamins and carotenoids Therapeutic agents presents in plantspossessignificance and extraordinary biological activities.

Therapeutic herbs displayed more antioxidant activity and contained fundamentally larger amounts of phenolics and other secondary metabolites than regularly known vegetables and fruits those are considered as rich sources of common dietary antioxidants (Beech *et al*., 2017 : Gulfraz *et al*., 2008)

Extract from the leaves of Eremophila species (Myoporaceae) were the most dynamic, with Eremophiladuttonii displaying the best action (against Gram-positive microscopic organisms). The most dynamic antibacterial plants against both gram-positive and gram-negative microscopic organisms were Thymus vulgaris and Thymus origanium. The two extract from similar plants indicated diverse exercises; the organic extract demonstrated the same or more noteworthy activity than the watery extract . Out of the 14 plants examined, Fragariavirginiana Duchesne, Epilobiumangustifolium L. furthermore, Potentilla simplex Michx. shown solid antifungal potential.Fragariavirginiana had some level of action against the majority of the parasitic pathogens. AlnusviridisDC.,Betulaalleghaniensis Britt. what's more, SolidagogiganteaAit. additionally showed a huge level of action against a significant number of the yeast separates (Briskin, 2000).

A basic oil portion from Canella winterana was additionally tried. The antimycobacterial movement of these substances was tried against Mycobacterium tuberculosis, M.aviumand M. kansasaii utilizing the Middlebrook 7H11 agar medium, the Bactec 460-TB radiometric system, and assurance of bacterial suitable tallies. Three mixes, to be specific ibogaine, voacangine and texalin, demonstrated antimycobacterium action (Burt, 2004; Cai *et al*., 2004).

The extracts were taken from Urticamembranacea (Urticaceae), Artemesiamonosperma (Asteraceae), and Origanumdayi post (Labiatae). Each of the three plant extracts showed dosage and time-subordinate killing abilities in different human inferred tumor cell lines and essential societies set up from patients' biopsies. The executing action was particular toward tumor cells, as the plant extracts had no impact on essential societies of solid human cells (Cushine and Lamb, 2011; Essawi and Srour, 2000) .

From the tried raw extract, Inulagraveolens, Salvia dominica, Conyzacanadiensis and Achilleasantolinashowed powerful antiproliferative activity and the action dwelled in the

chloroform/ethanolic separates. The most dynamic plant was I. graveolens with an IC50 of 3.83 µg/ml .

Three unique extracts (oil ether, ethylacetate, and methanol) from each plant species, were tried towards KB, HCT-15 COLADCAR and UISO-SQC-1 cell societies. The outcomes demonstrated that three plants Colubrinamacrocarpa (Cav.) Wear (Rhamnaceae), Acacia pennatula (Schltdl. furthermore, Cham.) Benth (Leguminosae) and Hemiangiumexcelsum (HBK.)Smith (Hippocrateaceae), displayed essential cytotoxic action demonstrating a specific level of selectivity against the tried cells in culture (Graf *et al.*, 2010; De Souza *et al.*, 2007) .

Extracts taken from 10 South American therapeutic plants (Baccharistrinervis, Baccharisteindalensis, Eupatorium articulatum, Eupatorium glutinosum, Tagetespusilla, Neurolaenalobata, Conyza floribunda, Phytolaccabogotensis, Phytolaccarivinoides and Heisteriaacuminata) were selected for in vitro antiviral action against herpes simplex write I (HSV-1), vesicular stomatitis infection (VSV) and poliovirus compose 1. The most strong hindrance was seen with a fluid concentrate of B. trinervis, which restrained HSV-1 replication by 100% at 50– 200 µg/mL, without indicating cytotoxic impacts. Great exercises were likewise found with the ethanol concentrate of H. cuminata and the fluid extract of E. articulatum, which showed antiviral impacts against both DNA and RNA infections (HSV-1 and VSV, individually) at 125– 250 µg/mL (Foster and Duke, 2000; Feng *et al.*,2011)

Five plants which have been utilized for the treatment of ailment, joint pain and edforgout in the conventional prescription of Saudi Arabia, were assessed for their anti inflammatory properties. Of these the ethanolic extract of Capparisdeciduas and the fluid concentrate of Capparisspinosa were found to have critical mitigating action against carrageenan actuated oedema in rats (De souza *et al.*, 2007)

Plants are equipped with many chemical constituents. There are two types of plant constituents known as primary and secondary metabolites.Essential metabolites are associated with the essential digestion of plants. A portion of the essential metabolites are nucleic acids, starches, lipids, proteins and chlorophyll. Plants store the overabundance of essential metabolites that are discovered either in stem, leaves or roots, and are .Auxiliary metabolites incorporates synthetic constituents which are not being utilized in essential digestion. In the past they were viewed as excretory items or finished results. Presently their significance has been acknowledged by the

solution. These constituents are presently viewed as therapeutically critical constituents. Optional metabolites assume a vital part of safeguard for the plants. They shield the plant from bugs and furthermore fend off the herbivores from them (Dynesius and Jansson, 2000).

The Pinaceae (pine family) are trees or bushes, including a considerable number of the outstanding conifers of business significance, for example, cedars, firs, hemlocks, larches, pines and spruces.Pine Bark Band: The inward bark can be used as a germ-free band for cuts and scraps. It is applied to wounds with channel tape, handkerchief, or cordage(Essawi and Sour, 2000; Hussain *et al*., 2008; Keeley, 2012).

Restorative Properties include: Germ-free, astringent, provocative, cell reinforcement, expectorant, high in Vitamin C for colds, influenza, hacks, clog, and even scurvy. Shikimic corrosive, the primary fixing in Tamiflu, is reaped from pine needles in Asia.

Pine Needle Tea: Pine needle drink is taken to remove the valuable stuff when one feel influenza like manifestations in body . Essential oils have been widely used since long for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal and cosmetic applications. Nowadays they are widely used in pharmaceutical, sanitary, cosmetic, agricultural and food industries (Isman *et al.*, 2011).

Various strategies are utilized so far for the extraction of fundamental oil from plant material, for example, steam refining, dissolvable extraction and so forth. The fundamental oil extraction strategy can be isolated into two noteworthy classes in light of temperature utilized for extraction i.e extraction at low or high temperature and extraction at room temperature. The strategies are headspace gathering of volatiles, hydro distillation, steam distillation , soxhletextraction and water and steam refining (Javanmardi *et al.*, 2003; Ji et al., 2009). The possible role and mode of action of these natural products is discussed with regard to the prevention and treatment of cancer, cardiovascular diseases including atherosclerosis and thrombosis, as well as their bioactivity as antibacterial, antiviral, antioxidants and antidiabetic agents.

MATERIAL AND METHODS

Collection and preparation of samples

The samples of needles leaves of three different pinus trees were collected in fine plastic bags duly labeled with date and areas of collection of samples. The collection of samples based on ethnobotanical uses by inhabitants of rural areas of Abbottabad regions. The samples were distinguished by a taxonomist at the Department of environmental Sciences, COMSATS University Islamabad (Abbottabad campus) and voucher specimen (No. 135) was deposited for future reference. Around 3 kg of plant material were moved to Biochemistry research laboratory, Division of Science, COMSATS University Islamabad,, Abbottabad. The needles leaves of the pinus species were used for the extraction of various phytochemicals and oils as well as determination of various bioactivities .

Plant materials were washed with demineralized water to evacuate undesirable materials including dust. Plants samples were shade and sun dried followed by oven dried for over night at 50 °C . The dried examples were ground with electric grinder, sieve 80mesh and saved in plastic bags at lower temperature till further uses.

Proximate analysis

The moisture contents of leaves were determined by weighing before after heating high temperature for overnight. Crude lipid was determined by using 5 gram of samples in 100 ml of ether through soxhlet apparatus , where as dietary fiber and ash contents of needles leaves were determined by using AOAC methods.

Determination of total phenols:

The concentration level of phenol in need leaves sample was determined in different solvents and amount was quantified by using method reported by various authors (Ji *et al*., 2009; Koehan and Carter, 2005). Briefly 100 µl of extract was diluted with 3 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent was added. After 3 min, 2 ml of 20% sodium carbonate was added and the contents were mixed thoroughly. The color was developed and absorbance was measured at 650 nm in spectrophotometer (Shimadzu UV-1800). Gallic acid was used as standard and

different concentrations of Gallic acid were used to draw standard curve (R^2 = 0.9926). The amounts of total phenolics were expressed as gallic acid equivalents (GAE) mg/100g of dry matter (Monfalouti *et al.*, 2010; Liang *et al.*,2008).

Determination of flavonoids

The extract of sample was prepared in five solvents . The flavonoid contents of extracts were quantified by using method reported by Husain et al.(2008). An aliquot (1 ml) of extract or a standard solution quercetin (4mg/ml) was added to 10 ml flask containing 4 ml distilled water and 0.3 ml 5% $NaNO_2$ was added. After 5 minutes, 0.3 ml of 10% $AlCl_3$ was added and after 6 minutes, 2 ml of 1M NaOH was further added and the total volume was made up to 10 ml with distilled water. Absorbance was read at 510nm with a spectrophotometer and the concentrations of flavonoid in the samples were expressed as mg quercetin equivalent /g of sample (Krymov, 2002).

Estimation of tannins:

The total concentration of tannins in different leaves extract was determined by Folin Denis method. The measurement of blue color formed by the reduction of phosphotungstomolybidic acid by tannins is used for colorometric determination of Tannins and 7.5 ml of distilled water was added to 1.0 ml of extract and standard solution of tannic acid. Then 1ml ofsodium carbonate was added following addition of 0.5 ml of Folin Denis reagent. The solution was diluted with distilled water up to 10 ml and absorbance was measured at 700 nm . The total tannic acid concentration was expressed as mg of tannic acid equivalent per gram of extract (Muthu *et al.*,2006).

Analysis of flavonoids with High Performance Liquid Chromatography

The HPLC analyses were performed by using shimadzu system (Tokyo, Japan) C18 column (250 mm × 4.5 mm, 5 m) gradient pump, UV/Visible detector. The compounds were eluted with a gradient elution of mobile phases (Acetonitrile and 0.1% phosphoric acid ; 36:64), and the injection volume for all samples was 20µl. Flavonoids were monitored at 280 nm and 285 nm at a flow rate of 1 ml/min. The quercetin was used as standard and all determinations were performed in triplicates (Newman *et al.*, 2000).

Extraction of oils by Soxhlet methods

Steam refining is a partition procedure utilized by cleanse or separate temperature delicate materials,like normal sweet-smelling compounds. Steam or water is added to the refining contraption to bring down the breaking points of the mixes. The fundamental rule of steam refining is that its permits a compound or blend of mixes to be separated at a temperature impressively underneath that of the breaking point of the individual constituent .basic oils contain substances with bringing down breaking points and within the sight of steam or bubbling water, these mixes are volatilized at a temperature of around 100°C (Masamgo, 2005).

The fundamental oil from crisp aeronautical parts of the plant was separated utilizing strategy effectively depicted (116) with a few adjustments. The ethereal parts of the chose plants were cut into littler pieces with the assistance of blade and scissors. The little bits of plant material could yield more noteworthy biomass that is effortlessly removed with high effectiveness .subsequent to changing over into little pieces, the heaviness of elevated parts of plant was controlled by utilizing an electric advanced adjust. The measured plant material was then raced into a spotless vessel of refining mechanical assembly. Roughly 2000ml refined water was added to the vessel and afterward put on warming mantle. The vessel was then associated with a condenser that was cooled by frosty faucet water. The stream of water through a condenser was begun and afterward the subsequent blend was bubbled for 3 hrs, the distillate containing unstable mixes was gathered in an isolating pipe in the wake of going through the water condenser. Layer of oil at the highest point of water in isolating cup obviously uncovered the nearness of basic oil. At that point, basic oil coasting on distillate was isolated and the rest of the distillate was utilized to isolate oil from it by fluid – fluid extraction (Newman *et al*., 2003).

The plug of isolating channel was opened for at some point to discharge weight. The shaking procedure was rehashed atleast three times and isolating channel was hanged in a stand holder. Following a couple of minutes a reasonable layer of hexane containing basic oil was framed over the water which was isolated in another flagon. A similar system was rehashed three times by including 70 mL of hexane in the leftover distillates. A few hints of water still present in hexane extricate was expelled by expansion of some measure of anhydrous magnesium sulfate to hexane separate. Hexane extricate containing magnesium sulfate was then sifted in a pre-weighed round base flagon (WHO, 2005; Palombo and Semple,2001).

Under reduced pressure most of the hexane was evaporated by using rotatory evaporator. On analytical balance oil free from hexane was weighed to find yield of oil by dividing the extracted oil mass by the mass of plant used for the extraction of oil . By means of specific glass adopter .round bottom flask having hexane extract was connected to rotary evaporator. To collect the hexane after evaporation collecting flask was connected to the condenser of rotatory evaporator. At the beginning, the vaccum of rotatory evaporator was set at 200 mbe at 25℃. when the evaporation from flask get stable right after 3 mins the pressure was decreased to 100 or 80 nbr. The process was continued till all the hexane get evaporated from round bottom flask. Then the yield (%) of pure essential oil was calculated by dividing the mass of essential oil to the mass of fresh plant material used for the extraction of oil (Petrovska, 2012).

Briefly samples were waterlogged overnight and washed thoroughly to eliminate the pulp. The fruitssamples were air dried at room temperature crushed into powder from and used for the estimation of oil contents by using AOAC Official Method. Total 2 grams of sample by addition of appropriate amount of ether in Soxhlet apparatus was used for extraction of oil . The reaction continued for 16 hours and results were expressed as percentage of dry weight of fruit.

Analysis of oil for fatty acids with GC-MS

Fatty acid methyl esters: pinus needles leaves oil in n-heptane (0.20 g per 2 mL) was transmethylated using a cold solution of KOH (2 mol L−1) (200 μL) and methyl esters (FAME) was analyzed. The composition of extracted oil was examined by utilizing Gas Chromatography-Mass Spectrometry. The hawlett Packard framework was utilized to analyze the sample. The 6890N was outfitted with DB-5 section (30 m length, 0.25mm inner distance across and 0.25 µm stationary stage film thickness) and combined with a HP 5973 Mass spectrometer (MS; Agilent Advances Inc. USA). The injector was worked at 235 ℃. The stove temperature of GC was customized as: the underlying temperature of broiler was 40 ℃ and it was kept up for 2 min, the temperature was raised from 40 to 450 ℃ at a warming Rate of 4℃ and stayed at higher temperature for 8 mins. Exceptionally unadulterated Helium gas was utilized as portable stage with a steady stream of 1mL/min.The weakened arrangement of fundamental oil was infused with volume of 1 µL in split less mode. Mass spectra was performed by an electron ionization

framework worked at the ionization vitality of 70 eV. The particle source temperature of mass spectrometer was set at 180°C and the deferral for dissolvable was 5 mins. The mass spectra filter extend was 30-400amu. GC top territories were utilized to register the rate structure of an example without utilizing remedy factors. The distinguishing proof of fundamental oil constituents was at first completed by contrasting mass spectra of a compound and NIST-2008 MS library. As a second step, the maintenance lists of isolated mixes were resolved in respect to the maintenance times of standard C9 to C24 n-alkanes at a similar GC-MS parameters utilized for the basic oils. The figured maintenance lists of mixes were contrasted and the distributed information for the assurance of elution request and distinguishing proof of mixes. At last, the distinguishing proof of compound was accomplished by co-infusion

Bioactivity assessment of *Pinus* needles leaves

Antioxidant activity

The antioxidant activity of plant extracts was carried out according to method earlier reported (Javanmardi *et al.*,2003) Therefore, for determination of antioxidants, following bioassays were used.

DPPH scavenging bioassay

DPPH scavenging activity was done according to method with some modifications. This method was based on the ability of antioxidant to scavenge 1, 1-diphenyl-2-picryl hydrazyl (DPPH) action radical. In a falcon tube, 100 µL of the sample solution was taken and then 4mL of DPPH solution (0.1 mM) was added and mixture was vortexed vigorously. Then the mixture was incubated with Aluminium foil to avoid heat exposure in the dark for 30 min at room temperature. UV-Vis spectrophotometer was used to measure the absorbance of the solution at 517 nm. Percentage inhibition was calculated by

DPPH scavengingaction was carried out by technique with a few alterations. This strategy was depended on the capacity of cell reinforcement to rummage 1, 1-diphenyl-2-picryl hydrazyl (DPPH) activity radical. In a falcon tube, 100 µL of the example arrangement was taken and afterward 4mL of DPPH arrangement (0.1 mM) was included and blend was vortexed energetically. At that point the blend was brooded with Aluminum thwart to maintain a strategic distance from warm introduction oblivious for 30 min at room temperature. UV-Vis

spectrophotometer was utilized to quantify the absorbance of the arrangement at 517 nm. Rate hindrance was ascertained by

$$\% \text{ Inhibition} = A_{blank} - A_{sample} \times 100 / A_{blank}$$

ABTS scavenging bioassay

The ABTS radical scavenging bioassay was carried out by already reported method. The ABTS radical cation was prepared by mixing 5 mL of a 7 mM aqueous ABTS solution to an about 88µL of 140 mM potassium per sulphate by making the final concentration of solution to be $0.7cm^{-1}$. Then the plant extracts were prepared in ethanol to a volume equal to 25 µL. 10µL of sample solution was added to the reaction mixture and final percentage reduction in absorbance was measured at 730nm. The expected final absorbance was assumed to be 20-80% decreased as compared to the initial absorbance of reaction mixture.

Scavenging of H_2O_2

Scavenging of H_2O_2 was determined by using method reported and briefly about 4 mM of H_2O_2 (0.6 mL) solution was added to 4 mL of extract and incubated for 10 min. The absorbance of solution was measured at 230 nm against a blank solution via spectrophotometer method. Free radical scavenging potency as determined from %age of H_2O_2. Lower of H_2O_2 indicated strong free radical scavenging activity (Koehn and Carter, 2005).

Brine shrimp cytotoxicity bioassay

Hatching of shrimps

Brine shrimp eggs were hatched in a shallow rectangular dish (22 x 30 cm) filled with artificial sea water that was prepared with a commercial salt mixture and double distilled water. A plastic divider with 2 mm holes was clamped in the dish to make two unequal compartments, the eggs (50 mg) were sprinkled into larger compartment which was darkened while the smaller compartment was illuminated. After 48 hrs, the phototropic napulii were collected by pipette from the illuminated side, which was separated by the divider from their shells (Saeed *et al.*,2012).

Twenty shrimps were transferred to each sample vial using pipette and 5mL artificial sea water was added. The napulii can be counted in the stem of pipette against a lighted background. A drop of dry yeast suspension (3 mg in 6mL artificial sea water) was added as food to each vial (Solowey *et al*., 2014). The vials were maintained under illumination. Survivors were counted with the aid of 3 magnifying glass and after 24 hrs percent death at each dose and control were determined. In each case, where control deaths occurred, the data were corrected using Abbott's formula:

$$\% \text{ Death} = [\text{ Test} - \text{Control}] / \text{Control} \times 100$$

Antibacterial activity

Preparation of inoculum

Suspension of organisms was prepared as per McFarland's standard and 24 hours old culture was used for the preparation of bacterial suspension. Bacterial strains (colony) were picked in eppendorf tubes with the help of inoculation loops and placed in 37°C incubator for 30 minutes. Each bacterial colony collected was then mixed in 3mL distilled water and then shaked vigorously or vortexed and optical density was checked and made equal to 0.5 by using spectrophotometer at 600 nm wavelength (Sher, 2004).

Test organism: Bacterial strains

The plant extracts of three Pinus species were screened against five bacterial strains by following the method reported (Tsao and Liu, 2007). The strains of Gram +ve bacteria such as Staphylococcus aureus (KX262674) and Bacillus cereus (KX262674) and Gram –ve bacteria Escherichia coli (ATCC 10536), Salmonella typhi (ATCC 6539) and Pseudomonas aeruginosa (ATCC 9027) were included in this study. Each bacterial colony was mixed with 3 mL sterilized water, vortex ed and optical density was made equal to 0.5which means culture contained no. of colony forming units (CPU) in the range of $10^7 – 10^8$ per ml of suspension depending upon bacteria. These strains were grown in nutrient broth and then cultured on nutrient agar for their maintenance and were stored in refrigerator at low temperature for reculturing before use in experiment as reported earlier.

Antibacterial activity of Pinus essential oil

Antibacterial activity of essential oils extracted from was assessed using agar well diffusion method. Nutrient agar was prepared by pouring accurately weighed 13 g/L of nutrient broth and 14 g/L of agar technical in 1L of distilled water in reagent bottle and was mixed thoroughly that it may get mixed. Then the mixture was autoclaved with the temperature at 110°C for 60 mins. Then agar plates were poured by 30-35 mL of autoclaved nutrient media, covered and sealed with parafilm. The covered plates were allowed to rest for an hour so the agar can solidify and then petri plates were placed in an incubator at 37°C for overnight. 200 µL of each bacterial strain was evenly spread on surface of petriplates using glass spreader. Then four uniform and equidistance wells were made with 6mm (diameter) cork borer in each plate. Each well was filled with 30µL of essential oil test solution and 2 replicates of each test petri plate were made. Hexane was used as negative control and streptomycin (1 mg/ mL) was selected for positive control as the reference for all bacterial strains. After half an hour, the plates were placed in incubator at 37°C for 24 hrs. The size of zone of inhibition of each well in a petri plate was individually measured in millimeters by using a scale at four different places around the inhibition zone circle (Shinwari, 2010).

Anti mycobacterium activity

The two drug resistant strains of *Mycobacterium tuberculosis* ,bg 206 and bg 1972 along with a sensitive strain H37Rv were used in this experiment. Inoculum

of all strains were prepared by using method described by Cushine and Lamb(2011; Maryam *et al*., 2021).From dilution (10^{-2}) of each strains of *Mycobacterium tuberculosis* suspensions, about 60µl was streaked on the LJ slants using loop with 3mm external diameter. The crude extract at various concentrations of 05 mg/ml, 10 mg/mL and 50 mg/mL were separately incorporated in the medium and this process was performed for all extracts. Antimycobacterium activity was done according to the methods of (Gulfraz *et al*., 2008; Roy *et al*., 2994; Rios, 2010).

Statistical analysis

Data obtained were analyzed statistically by using one way ANOVA and results were expressed in form of mean, standard deviation and percentage values.

RESULTS AND DISCUSSION

Proximate analysis

Results of proximate analysis indicates that pinus*Roxburghii* contained higher quantity of moisture (18.5 ± 2.6 %), dietary fiber (24.6 ± 2.8 %), ash (2.4 ± 0.5 %) ,crude lipid (5.6± 0.7 %) and carbohydrates (45.5 ± 2.5 %) as compared to other extracts (Table 1).

Table1. Proximate analysis (%) of pinus needles leaves

Extracts	Moisture	Dietary fiber	Ash	Crude Lipid	Carbohydrates
PinusRoxburghii	18.5±2.6	24. 6±2.8	2.4±0.5	5.6 ± 0.7	48.5 ±2.5
PinusWallichiana	14.6±1.5	23.5±1.6	2.2 ± 0.8	4.9 ± 0.5	54.5± 1.6
cedrus deodar	14.8±1.2	21.8±1.5	2.6±0. 7	3.5 ± 1.2	57.3 ±1.5

Mean ±SD (n=3)

Analysis of phytochemicals

Quantitative analysis of extracts shows that the amount of phenole, flavonoids and tannins (Table 1). Result s has shown that the extract from needles of *PinusRoxburghii*carries the higher amount of phenol (7.26 mg/g) followed by *Pinus Wallichiana*which contain 5.42 mg/g of phenol while *cedrus deodar* has 5.86 mg/g of phenol content in needles extracts.. The amount of flavonoids was maximum in plant

extract of *PinusRoxburghii* (14.53 mg/g) whereas,*PinusWallichiana* and *cedrus* deodar contained 8.56 and 5.26 mg/g. Similarly the yield obtained from tannins was greater in *PinusRoxburghii(4.36* mg/g *)* followed by 3.46 mg/g in *PinusWallichiana* extract while 2.68 mg/g tannins were found in extract of cedrus deodar (Roy *et al*., 2004).

Table 2 Chemical analysis of *pinus* needle leavesof*PinusRoxburghii* (chir pine)

Constituents	**n-hexane**	**Ethanol**	**Methane**
Total phenol mg/g	2.06±0.27	4.28±0.72	7.26±1.52
Total flavonoids mg/g	4.13±0.16	5.15±1.38	14.53±2.45
Total tannins	1.33±0.25	2.87±0.54	4.36±1.23

Mean ±SD (n=3)

Table 3. Chemical analysis of *pinus* needle leaves of *PinusWallichiana*

Constituents	**N-hexane**	**Ethanol**	**Methane**
Total phenol mg/g	2.23±0.28	3.78±0.62	5.42±2.83
Total flavonoids mg/g	3.18±0.82	5.65±2.31	8.56±1.21
Total tannins	1.12±0.32	2.35±0.38	3.46±1.58

Mean ±SD (n=3)

Table 4. Chemical analysis of *pinus* needle leaves of *cedrus deodar*

Constituents	N-hexane	Ethanol	Methane
Total phenol mg/g	2.96±0.28	4.13±0.82	5.86±1.05
Total flavonoids mg/g	4.35±0.75	6.45±1.32	6.26±1.76
Total tannins	1.65±0.82	2.15±0.27	2.68±0.41

Mean ±SD (n=3)

Assesment of yield of essential oil

The extraction of essential oil was carried by hydro distillation from needles of *Pinus Roxburghii*, *Pinus Wallichiana* and *cedrusdeodara.* Needle extract of *PinusRoxburghii*and *PinusWallichiana* were rich in essential oil and yielded 1.92% and 1.68% essential oil respectively. *Cedrusdeodara*also showed good yield however, the amount was less as compared to other two *pinus*species (Table 2-4).

Table 4. Percentage yield of essential oils

Latin name	Family	% yield of essential oil
PinusRoxburghii	Pinaceae	3.92±0.28
PinusWallichiana	Pinaceae	3.68±0.52
cedrusdeodara	Pinaceae	2.25±0.05

Mean ± SD after triplicate analysis

HPLC analysis of flavonoids

Methanolic extracts of pinus needles was analyzed with HPLC and flavonoids were monitor at

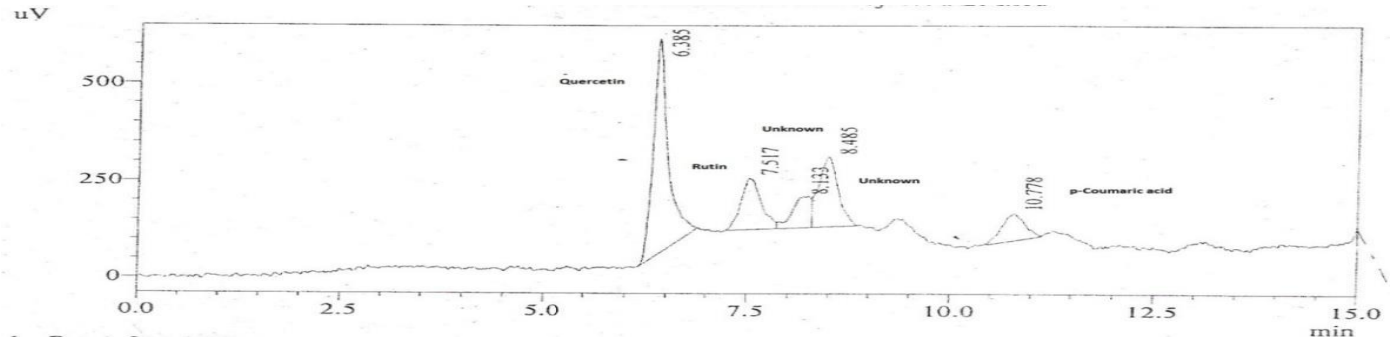

Figure 1. Analysis of flavonoids (quercetin) with HPLC at 750 nm wavelength

Analysis of oil by GC- MS

Oil extracted from various samples of pinus needles was analyzed by GC –MS .It was found many fatty acids were present (Table 5).

Table 5 Fatty acid contents of oil from cedrusdeodara needles leaves analyzed by GC-MS

Name	**RT**	**%compounds**
1-DODECANOL, 2-OCTYL-	8.801	0.008304
TETRAPENTACONTANE, 1,54-DIBROM	11.042	53.84524
1(2H)-NAPHTHALENONE, 6-(1,1-DI	21.181	41.54379
1-DODECANOL, 2-HEXYL-	32.356	0.013741

There were four compounds of fatty acids detected from oil of cerdusDeodara by GC-MS . However, two compounds like tetrapentacontane (53.84 %) and Napthahalenone (41.5%) were present with higher concentration

Table 6 Fatty acid contents of oil from pinus Roxburgii needles leaves analyzed by GC-MS

Name	RT	Area	%composition

OCTADECANE, 1-CHLORO-	22.422	11131664384	96.51861
1-DODECANOL, 2-OCTYL-	33.801	25381554	0.220074
1-DODECANOL, 2-HEXYL-	34.807	6787248.5	0.05885
HEPTACOSANE, 1-CHLORO-	36.943	10392788	0.090112
17-PENTATRIACONTENE	37.098	3289870.25	0.028525
1-PENTACONTANOL	37.538	744749.688	0.006457
TETRAPENTACONTANE, 1,54-DIBROM	42.08	53473556	0.46365

There were seven compounds of fatty acids detected from oil of pinusroxburgii by GC-MS . However, compound like OCTADECANE, 1-CHLORO- (96.51) is present with higher concentration

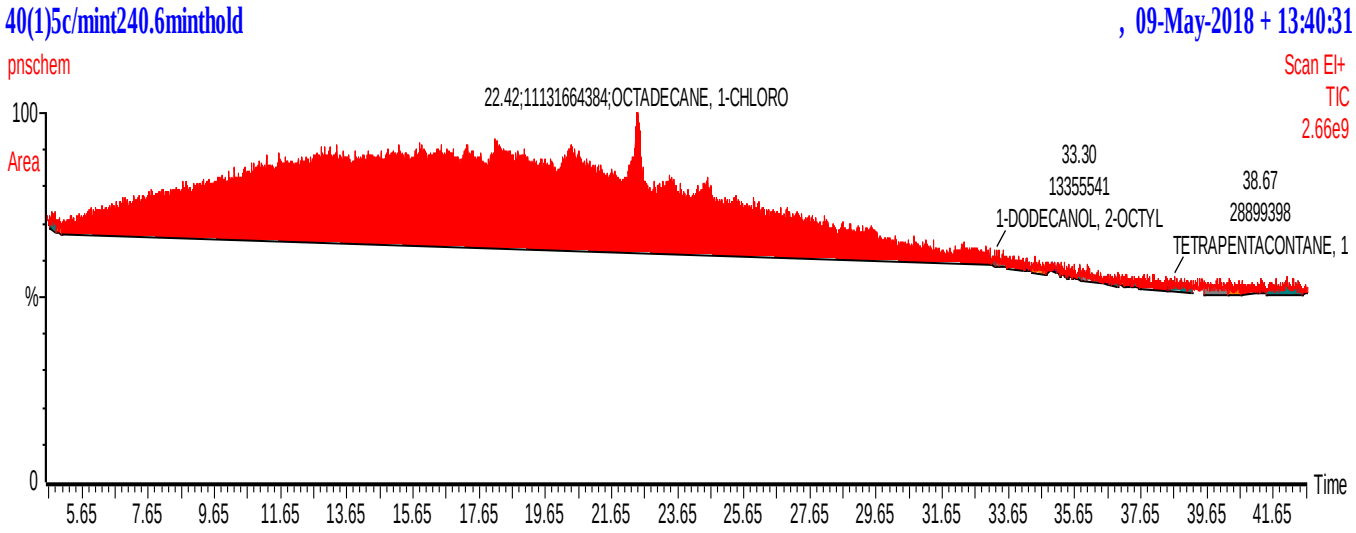

Figure 2. GC – MS analysis of pinus needl leaves

Table 7 Number of Carbon atoms of fatty acid found in pinus needles leaves

Fatty acid	P. R (%)	C
C18 :O	96.5	0.08
C 15: 0	0.64	
C 20 :0	0.22	53.84
C 27:0	0.31	0.13
C35: 0	0.62	-
C 54: 0	0.46	-
C8 :0	-	41.54

Bioactivities of pinus needles leaves

Antioxident activities of leaves extract

In order to find out antioxidant activities, the extracts from needles of Pinus were employed for the DPPH, ABTS and H_2O_2scavenging bioassays. The scavenging activity of the needles extracts was much greater in H_2O_2 scavenging assay as compared to ABTS and DPPH scavenging assay

Table 8 . Antioxidant effects of leaves extracts of *PinusRoxburghii* (chir pine) *(*IC50 values µg/ml)

Extract 100 µg/ml	DPPH	H_2O_2	ABTS
Ethanol	45.17±3.26	58.54±5.26	45.32±2.81
Methanol	25.38±4.15	42.56±3.15	39.46±2.28
N hexane	52.18±1.36	61.52±4.85	48.24±1.35
Ascorbic acid	9 .65±2.52	7.62 ±1.36	16.25±2.38

Gallic acid	6.34±1.32	5.65±1.25	8.26±1.36

Means ± SD, (n = 3).

Table 9. Antioxidant effects of leaves extracts of *Pinus Wallichiana* (IC50 values µg/ml)

Extract 100 µg/ml	**DPPH**	**H_2O_2**	**ABTS**
Ethanol	36.17±1.25	45.69±4.15	39.45±2.16
Methanol	27.28±1.48	38.21±3.17	32.89±4.36
N hexane	45.16±1.51	48.93±6.53	53.27±3.25
Ascorbic acid	12.29±1.43	9.12 ±2.36	12.35±1.25
Gallic acid	8.35±1.36	7.68±1.25	8.24±1.32

Means ± SD, (n = 3), whereas $^{\alpha}$ = $p<0.01$,Þ= $p<0.05$.

Table 10 .Antioxidant effects of fruit extracts of *Cedrusdeodara* (IC50 values µg/ml)

Extract 100 µg/ml	**DPPH**	**H_2O_2**	**ABTS**
Ethanol	47.16±3.64	53.65±2.11	48.15±2.13
Methanol	38.36±4.58	42.23±2.38	45.68±4.35
N hexane	54.14±5.64	48.37±3.61	48.29±1.26
Ascorbic acid	9.22±1.38	8.05 ±2.38	12.65±1.62
Gallic acid	8.16±1.32	7.53±1.26	9.16±1.36

Means ± SD, (n = 3), whereas $^{\alpha}$ = $p<0.01$,Þ= $p<0.05$.

Antibacterial activity of essential oil of*P. Roxburghii , P. Wallichiana and C.deodar was* tested againstthe strains of Gram+ bacteria such as *Staphylococcus aureus(KX262674)* and *Bacillus cereus(KX262674*) ;and Gram – bacteria *Escherichia coli (ATCC 10536)* and *Salmonella typhi (ATCC 6539)*. The results showsthat essential oils has inhibited growth of bacterial strain significantly. The zone of inhibition provided by *P. Roxburghii* for S. auresu (17.6 ± 0.6 mm), B. cereus (16.4 ± 0.9 mm), S trphi (21.5± 0.3 mm) and E. Coli (21.8 ± 0.4mm) were relevatively higher as compared to other methanolic plant extracts as well as standard antibiotic used (Table 11).

Table 11 Antibacterial activities of various methanolic pine needles extracts ; Zone of inhibition in mm

Extracts	***S. aureus***	***B. cereus***	***s.typhi***	***E.coli***
P. Roxburghii	19.6±0.6	17.4±0.9	23.5±0.3	21.8±0.4
P. Wallichiana	17.6±0.6	16.4±0.9	21.6±0.3	21.3±0.2
Cedrusdeodara	16.2 ±0.5	18.3±0.8	17.5±0.4	16.2±0.5
Cefixime (Antibiotic)	18.3±0.6	21.2±0.3	18.2±0.4	17.8±0.6
Negative control	0.0±0.0	0.2±0.0	0.3±0.0	0.2±0.0

Results mean± S D after triplicate analysis (n=3).

Cytotoxiciy assessment of pinus needles leaves

Assessment of cytotoxic behavior of medicines pants used for drugs development are important which indicates that plant extracts is how much toxic to any cell. Results of brine shrimp cytotoxicity assay is given in table 12.

Table 12.Cytotoxicity screening of methanolic*P. Roxburghi* pine needles extracts (µg/ml)

Concentration (ug/ml)	**Total nupuli**	**Live after 24 hours**	**Death after 24 hours**	**% of death**
10	18	17	1	5.56

100	20	14	6	30.0
300	20	12	8	40.0
600	19	10	9	47.37

Values are Mean±SD ,(n=3) and significantly different ($P<0.05$); positive control are saline sea salt

Antimycorbaterium activity of pinus needles leaves

Medicinal plants offer a hope for developing alternate medicines for the treatment of TB. The present study was carried out to evaluate in vitro anti-tubercular activity of different extracts of pinus needles leaves extracts against different strains of mycobacterium tuberculosis (Table 13). Furthermore results obtained in our study showed higher percentage of inhibition results reported by other authors including Graf *et al*. (2010).

Table 13 .Anti-tuberculosis activities of *plant*extracts on LJ Media

Extracts	**Isolates**	**Mean CFU on media**				**Percentage Inhibition**		
		Control	5mg/ml	10mg/ml	50mg/ml	5mg/ml	10mg/ml	50mg/ml
Methanolic leaves extracts	H37Rv	140	36	2	0	74	99	100
	bg 206	150	49	23	0	67	85	100
	bg 1972	130	74	51	0	43	61	100
Methanolic leaves extract	H37Rv	140	30	0	0	79	100	100
	bg 206	150	45	20	0	70	87	100
	bg 1972	130	65	50	0	50	62	100

Methanolic leaves extracts	H37Rv	140	80	76	20	43	46	86
	bg 206	150	90	60	35	40	60	77
	bg 1972	130	86	64	40	34	51	69

Proximate parameters of any plant extracts give information regarding its suitability for used as feed or food, where as Phytochemicals present in the plants may be used as food and medicine. There is growing worldwide interest for characterization, isolation, the *in vivo* and *in vitro* assessment of biological activities of the phytochemicals to have beneficial therapeutic capacity for curing human from various illness. Therefore, variety of chemical compounds obtained by the tested plants impart significant anti proliferative, cytotoxic, antibacterial and anti-tuberculosis activities. In the present study, the plant extracts were assessed for the presence of flavonoids, total phenolics, saponin and tannin. The study indicates that the presence of these phytochemicals in all the three plants species contained the considerable amount of flavonoids, phenolics and tannins (Ullah and Khan, 2008). Phenols constitute the largest group of secondary metabolites, varying in size from a simple structure with aromatic ring to complex ones. Phenolic compounds, ubiquitous in plants are an essential part of human diet, and are of considerable interest due to their antioxidant properties. In the current study, higher TPC was found to be possessed by the plant extracts. Highest quantity of phenolics was found in P. Roxburghii as compared to others extracts analyzed Tannins (flavonoids) are astringent polyphenolic biomolecules that binds to and precipitates proteins and various organic compounds including aminoacids and alkaloids. Tannins are considered to be antimutagenic and this mutagenicity of tannins is related to their anti oxidative property (Tsao and Liu, 2007). The reported studies showed that tannins are present in lower concentration as compared to other phytochemicals in majority of plants, but our study reveal that plant species do possess tannins in them in considerable amounts. The phytochemical analysis showed thatP. Roxburghii contained higher quantity of tannins

Phytochemical prospection indicated the presence of different secondary metabolites. So the presence of significant amount of these important phyto-constituents bestow the plant with high medicinal activities like free radical scavenging and antimicrobial activities. The variation in

quantity of phytochemicals may be dependant upon both the chemical structure and the amount of individual compounds in plant material (Shinwari, 2010).

By comparison of chromatogram of GC-MS, the area of peaks of compounds was calculated and other required information was obtained by NIST library data base. The prominent peaks in the chromatogram of essential oil of indicates that pinus needles consist of some important fatty acids (Roy *et al*., 2004).

The variation in chemical constituents of essential oils might be due to the variation in the species of tree. The composition of oil directly affects the effectiveness of biological activities which have displaces difference in its constituents depending on the growing area whereas chemistry of oil is complex and variable as mentioned above. The seasonal changes may account in the variation in chemical constituents of plants grown at different parts of the world .

Chemical constituents with antioxidant activity present in plants determine the role of plants in prevention of many degenerative diseases. The human food supplements including herbs, contain higher amount of compounds that are capable of deactivating free radicals (Saeed et al.,2012). The plant extracts were assessed for their antioxidant potential by three methods that includes DPPH scavenging, ABTS scavenging and H_2O_2 scavenging bioassays. The methanolic extracts of the plants showed significant scavenging of free radicals. By comparing DPPH and ABTS bioassays results. It was assumed that the antioxidant potential of the three plant extracts is much increased in case of DPPH bioassay as compared to ABTS bioassay. Among the three plants, the antioxidant potential of *P. Roxburghii* was higher as compared to other plant extracts analyzed

DPPH and other scavenging bioassay revealed that free radical scavenging potential was present in plant extracts as been reported in literature by many authors might be significant antioxidant agent due to its excellent antioxidant activity for Reactive oxygen species (ROS). ROS and reactive nitrogen species (RNS) are some forms of activated oxygen and nitrogen respectively, which include free radicals such as superoxide ions, hydroxyl and nitric oxide radicals as well as non-free radical species such as hydrogen peroxide (H_2O_2), and nitrous acid (HNO_2). ROS and RNS have been the cause of more than 100 diseases which includes malaria, AIDS, heart diseases, stroke, diabetes and carcinogenity. It was reported thatpinus needles leaves have antioxidant as well as antimicrobial activity which may be attributed to the presence of various active secondary metabolites (Gulfraz et al., 2008).

Similarly in the current research work, has shown a considerable amount of antioxidant potential. However the studies conducted in the other parts of world revealed the good antioxidant potential and anti-inflammatory activity of *pinus needles leaves* .

Scavenging of H_2O_2 displayed by extracts of plants was dose dependent. The total radical scavenging activity (superoxide and hydroxyl radical) of whole plant extract pinus needles was found to be quite significant and showed effective scavenging activity in dose dependent manner and suppressed the production of H_2O_2 at the dose concentration 300 µg/mL which is a characteristic of chain-breaking antioxidants, and has been observed in oxidation of linoleic acid emulsion with extract . Flavonoids, phenolic acids and phenolic diterpenes are the examples of phenolic components with antioxidant properties. Scavenging of extracts may be characterized to phenolic content which is capable of donation of electron to H_2O_2, thus nullifying to water. Although reactivity o H_2O_2 is not much, even then it can periodically be cytotoxic by ascending hydroxyl radicals in the cells. Expulsion of H_2O_2 by food stuffs is too much necessary (Saeed *et al.*, 2012; Solowey *et al.*, 2014).

Antimicrobial activity of essential oil of *pinus needles* in methanol has been tested against Gram +ve and Gram –ve bacterial strains. The selected herbs have been used traditionally as a remedy for respiratory diseases like bronchitis, sinusitis, tuberculosis and common cold. These activities are mostly due to presence of oils like . The results indicate that essential oils and other phyochemicals present in plant extracts has exhibited significant antibacterial activity Results indicates that plant extracts has inhibited growth of bacterial strains So, it can be said that all the tested extracts are antibacterial in nature though the activity varies from extracts to extracts . The results obtained are in accordance with the reported findings (Ullah and Khan, 2008).

The antibacterial study indicates that the plant extractshave tremendous antibacterial activity at higher concentrations against various bacterial strains that are the major causative agents mainly of stomach problems. The finding of zone of inhibition was found to be higher than the results reported by other authors . Due to multidrug resistant strains of various bacterial strains new medicines are required to overcome tuberculosis in human population. Various plants have some active secondary metabolites those have wide range of application against such human disorders . Therefore there is need of continuous development of new and efficient methods to determine the susceptibility of isolates of mycobacterium tuberculosis in search for new novel

antimycobacterium agents from natural products of plants sources. Therefore in present study various solvents extracts of pinus needles leaves have provide remarkable anti mycobacterium activities indicating its usefulness control aliments of tuberculosis in human population (Vaghasova *et al.*, 2011) . Tuberculosis (TB) is an infectious disease mostly caused by mycobacterium tuberculosis mostly affects on lung but some cases also affects other parts of body . According to literature about one- third of world population is suffering TB , that is increasing at rate of 1 % per years (Tsao and Liu, 2007).

Cytotoxicity

Cytotoxicity refers to the ability of certain chemicals to destroy the living cells in the body. By a cytotoxic compound, healthy living cells either induce necrosis (accidental cell death) or apoptosis (programmed cell death). Brine shrimp lethality bioassay used forpinus needles leaves

indicates that all the plant extracts are very less toxic and are suitable to be used in folk medicine, which also indicates importance of *these* extracts for its application in pharmaceutical industry for development of drugs. P. Roxburghiishowed least toxicity as compared to other two *other*extracts during the bioassay. The toxicity of extracts was assessed which revealed that all of the plant extracts were less toxic towards shrimp's napulii at higher concentration (600 µg/mL) which confirmed their efficiency to be used in preparation of future drugs

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Volume 1, Issue 2. August, 2021. www.jbbt.org

PRODUCTION AND CHARACTERIZATION OF EXOGLUCANASE FROM *Phaeolusspadiceus*

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ABSTRACT

In economic sector, the main backbone of Pakistan is considered agriculture that produces a hug volume of waste material which has a very few economic uses. White rot Fungi play an important role in the production of hydrolytic enzymes like cellulases. In current study *Phaeolusspadiceus*.was used for the production of exoglucanase by Solid State Fermentation of wheat straw.*P.spadiceus* produced exoglucanase having high enzymatic activity during the fermentation of wheat straw under optimum physical and nutritional conditions. There was maximum exoglucanase activity after 72 h of fermentation, at 30°C temperature, pH 4.5, 70 % moisture level and 1 mL fungal inoculum. Further production was increased with glucose as

carbon source (5%) and peptone as nitrogen source (2.5%). The characterization of enzyme revealed that it has optimum temperature as 30°C and optimum pH as 4.5 using 1% Avicel aqueous solution as substrate. KCl, $ZnCl_2$, NaCl and $MnCl_2$ have positive effect on enzyme but Zn^{+2} ion has high positive effects on exoglucanase activity. The Km and Vmax were 34.48 mM and 175.43 μM/ ml/ min, respectively.

key words: Exoglucanase, *Phaelousspadiceus*, Wheat Straw, fermentation

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INTRODUCTION

Agriculture is the main backbone of Pakistan ineconomic point view. Open field burning of raw materials from agriculture cause climate change and pollution. The source of fermentable sugars, most abundant and renewable is lignocellulosic biomasson earth (Himmel *et a*l., 1999; Saleem *et al*., 2008 and Ahmed *et al*., 2009) and in nature are the most importantsource of fixed carbon (Yang *et al.*, 2007 and Ahmed *et al.*, 2009).Annual worldwide builds 10–50 billion tonslignocellulosic biomass from the plants (Sticklen, 2006 andPersad and Bisaria, 2014).The components of lignocelluloses consist of cellulose (~30 to 50%), hemicellulose (~20% to 35%), and lignin (~15% to 25%). The renewable biomass can be used to produce building blocks for many industrial products through acidic or enzymatic hydrolysis like the production of organic acids (Xia *et al*., 1999), ethanol (Brethauer *et al*., 2010) and other important chemicals (Mahmood *et al*., 2013).Renewable lignocellulosic materials with less cost will be used to produce bioproducts and bioenergy for the local economy and national energy protection (Zhang, 2008 and Zhang and Zhang, 2013).The lignocellulosic biomass isideal for enzymatic hydrolysis due to non-repressing by-products and nontoxic discharges (Persad and Bisaria, 2014) but high cost of industrial enzymes remains a barrier commercially.

In hydrolytic enzymes, cellulase is the most important group that catalyse the breakdown of β-1, 4 linkages thatappear in cellulose to give glucose. Cellulases are the group of extracellular enzymes including endoglucanase, exoglucanase and β-glucosidase. Exoglucanase acts on oligosaccharides that have reducing and non-reducing ends and releases cellobiose units, which

consist of glucose units more than two. Exoglucanase havetunnellike loop and three-dimensional(3D) structureat active sitefor interaction with substrate by hydrogen binding (Sinnott, 1997; Mahmood *et al*., 2013).Cellulases are important industrial enzymes and use in many industries like wine and brewery industry (Bamforth, 2009), textile industry (Karmakar and Ray, 2011), pulp and paper industry, food industry and bioethanol industry (Kuhad *et al*, 2011).

The researchhasfocus to improve the productivity of known enzymes, identify new and more active enzymes, and optimized characters of enzymefor lignocelluloses and less the production cost of enzyme (Merino and Cherry, 2007 and Zhang and Zhang, 2013). For enzyme production used different lignocellulosic materials are wheat straw (Norma and Guillermo, 2003; Yang et al., 2006 and Mahmood *et al*., 2013), wheat bran and corn cobs (Betini et al., 2009 and Mahmood *et al*., 2013)

Now-a-days, solid-state fermentation (SSF) is more used becauseof its advantages like lower investment spending, less cost of media for fermentation, quantity of better production, less energy needs and not essential requirement of manycontrols of fermentation parameters and low amount of waste production.

The capacity to degrade lignocellulose is high among fungi than bacteria.White-rot fungi that are responsible for efficient lignin degradation in wood decay processes. *Phaeolusspadiceus*is present in the heart wood of the tree. It is widely study for degradation of lignocellulotic biomass (Wheat straw) by secretion of extra cellular enzyme like exoglucanase.

MATERIALS AND METHODS

Substrate collection and preparation

Wheat straw was used as substrate for the growth of *P.spadiceus*to produce exoglucanase.Wheat straw was collected from the village of SamwalSharife, Mirpur Azad Kashmir, then air dry it for 20 days.Oven dried it for 24 hours at 60 ^{0}C for the removing of remaining moisture. After that, it was ground into powder form in the laboratory of Biotechnology, MUST, Mirpur Azad Kashmir. Powder of substrate was packed in airtight plastic jars before use in fermentation process.

Fermentation Organisms

Collection and Isolation of *P.spadiceus*

The sample of *P*.spadiceus was collected from the laboratory of Biotechnology, MUST, Mirpur Azad Kashmir. It was isolated by culturing on malt extract agar media in aseptic conditions. Slant was prepared from isolated pure culture on Malt Extract Agar (MEA) media. The slant and pure culture of *P.spadiceus* were stored at 4^{o}C in refrigerator for future use.

Preparation of Fungal Inoculum Media

For inoculum broth media was contained per litter; 3g of peptone, 20g of dextrose, 20g of malt extract and adjusted pH at 4.5. The inoculum media was autoclave at 121^{o}C and 15 psi for 15 minutes. After autoclave, the fungal spores were incubated into inoculum media from the culture plate under aseptic conditions. The flask containing inoculum media was placed in shaking incubator at 120 rpm at 30 ^{o}C for 2-3 days.

Fermentation Process

Flasks containing 5g of grinded wheat straw was moist with 2.5ml (50%) of d.H_2O, having pH 5.5 that maintained with the help of 1M HCL/NaOH and autoclave these flasks at 121^oC and 15 psi for 15 minutes. Each flask after autoclave was inoculated aseptically with 1ml of *P. spadiceus*inoculum and incubated these flasks at 30^oC for species days.

Sample Harvesting

After specified day, in each of the flask added 50ml of d.H_2O (pH 5.5) for the extraction of exoglucanase. These flasks were shake at 120rpm for 30 minutes in shaking incubator. The extra cellular enzymes dissolved in water, which was filtered with help of filter paper. Filtrate extract of enzyme, then centrifuge at 12000 rpm for 10 minutes to remove all spores and impurities. Supernatant was stored as a crude enzyme at 4^oC before preforming enzyme assay (Shafique et al., 2004; Mahmood et al., 2013).

Effect of Physical and Nutrients Conditions

Fungal growth and production were optimized by maintaining different conditions in order to gain maximum production of exoglucanase from *P. spadiceus*.During current study following conditions were optimized.

Effect of Fermentation Period

P. spadiceus growth was optimized for different fermentation time period from 24hr to 168hr. After different period, with gap of 24 hours, the harvested crude enzyme sample was subjected to enzyme assay.

Effect of pH

*P. spadiceus*was cultured, for the optimization of pH at ranging from 3.5 to 8.5 (six varying pH of distil water used as moisture content 50% into substrate) and adjusted with the help of 1M HCl/NaOH.

Effect of Moisture and Temperature Level

P. spadiceus was incubating at four different temperatures (25 ^oC, 30^oC, 35 ^oC and 40 ^oC) and three different moisture levels (30%, 50% and 70%) for 3 day (optimum).

Effect of Carbon and Nitrogen Sources

Carbon and nitrogen sources in different forms was used as substrate for the growth of *P. spadiceus* that increase the production of exoglucanase. For the carbon sources (Glucose, Fructose and Sucrose) and the nitrogen sources (Peptone, Urea and Ammonia Nitrate) were used during the current study.

Standard Curve

Cellobiose was used as a standard because exoglucanase act on the non-reducing end of cellulose and release disaccharide units (cellobiose). Different concentrations of standard were prepared in

0.0 µM-4 µM**.** Standard factor would be calculated by measuring absorbance of each concentration at 540nm.

Enzyme Assay

Avicel was used as substrate for exoglucanase (Sherief et al., 2010)**.** 1% solution of Avicel in distal water is used as enzyme assay. Following reagent are used for enzyme assay of exoglucanase.

DNS reagent: Dinitrosalicylic acid was used to form the coloured complexes with the product of exoglucanase. Five hundred ml solution of DNS was prepared by mixing of 5g of NaOH, 1g of phenol, 0.25g of Na_2SO_4, 91g of Roshelle salt and 5g of 3, 5-dinitrosalicylic acid**.**

Sodium citrate buffer: Five hundred ml. of buffer was prepared by mixing 3.88g of citrate acid and 1.91g of tri-sodium citrate in distal water with pH 4.8.

Procedure of Enzyme Assay

The activity of crude enzyme exoglucanase was observed by adding 1ml of crude enzyme, 1ml of 1% Avicel (substrate) and 1ml of Sodium citrate buffer having pH 4.8 in a test tube. Incubated the test tube at 30ºC for 30 minutes in the incubator. After 30 minutes added 3ml of Dinitrisalicylic acid (DNS) into each test tube to stop the reaction and tubes were placed in boiling water for 15 minutes. During boiling, DNS react with enzymatically hydrolytic product of exoglucanase to formed complexes. The concentration of these complexes was measured by spectrophotometrically at absorbance 540nm.

Enzyme Activity

One unit of enzyme activity defined as the amount of enzyme which released one micro-mole of glucose per minute.

Characterization of Exoglucanase

Purified exoglucanase was exposed to the characterization of different kinetic parameters.

Optimization of pH for Exoglucanase

To check the effect of pH on exoglucanase used two types of buffer (Sodium citrate buffer and Phosphate buffer). Enzyme assay was performed at different pH values of Sodium citrate buffer (pH-3.5, 4.5 and 5.5) and Phosphate buffer (pH-6.5 and 7.5).

Optimization of Temperature for Exoglucanase

Enzyme assay was performed at six different temperature levels e.g., 20 ºC, 25 ºC, 28 ºC, 30 ºC, 35 ºC, 40 ºC at pH 4.5.

Optimization of metal ions for Exoglucanase

Enzyme assay of exoglucanase was performed at five different metal ions solution ($CaCl_2$, KCl, $ZnCl_2$, NaCl and $MnCl_2$) and measured spectrophotometrically at 540nm. Metal ions interact with enzyme and change their structure and either enhance or inhibit their activity.

Effect of Substrate Concentration for Determination of Km and Vmax

For enzyme assay five different concentration of Avicel (2mM, 4mM, 6mM, 8mM and 10mM) were used to the determine Km and Vmax of the enzyme.

RESULTS AND DISCUSSION

The enzyme (exoglucanase) is one of important cellulose enzyme that is contributed to the degradation of large amount of wheat straw (cellulosic waste). The study of production of exoglucanase from fungus (*P. spadiceus*) and optimized it at different conditions will help to produce high quantity of enzyme that reduce environment pollution and produce appreciated things from resulted fermentable sugar. During the current study, different factors would discuss below that are optimized.

Effect of Physical and Nutrients Conditions

Effect of Fermentation Period

After Day-3 (72 hr.) of fermentation, *P. spadiceus*gave maximum production of exoglucanase (97.11 IU/mL/min) (Fig. 1). At day-3 production of exoglucanase was increased but after day-3 production of exoglucanase decreased due to depletion of nutrients and accumulation of waste materials. Current results show the resemblance with the resultsthat reported by Shafique et al. (Shafique *et al.*, 2004) for exoglucanase by fungal source (Mahmood *et al.*, 2013).

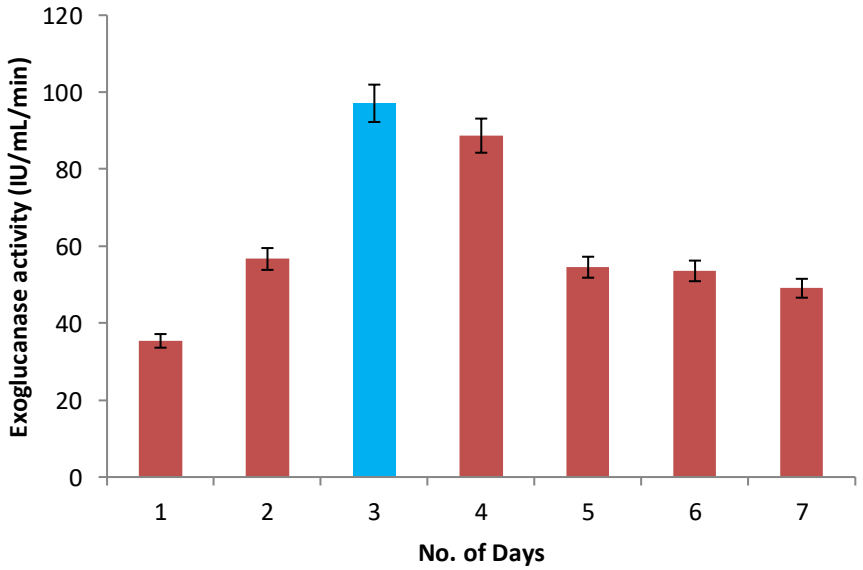

Figure 1. Effect of fermentation period for exoglucanase by *P. spadiceus*

Optimization of pH

P. spadiceus was produced maximum exoglucanase production (147.02 IU/ml/min)at pH 4.5 of fermentation (Fig. 2).After that, its activity decreased due to the acidic nature of enzyme and decreased in the stability of enzymes.

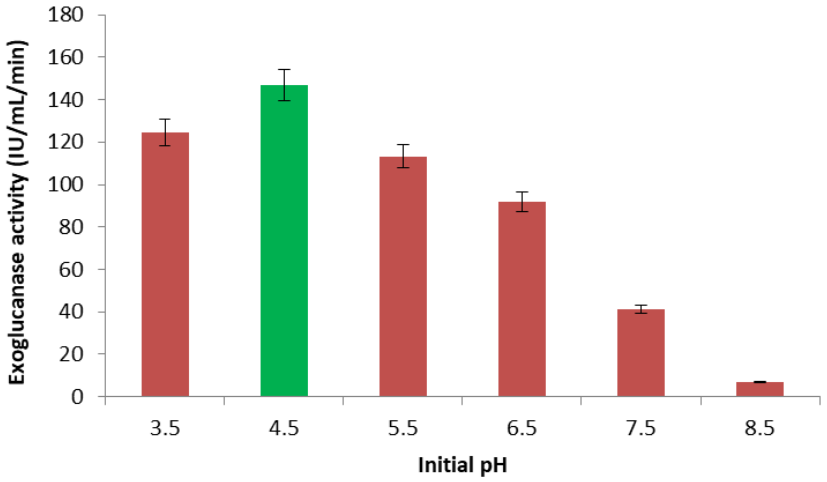

Figure 2: Effect of pH for exoglucanase by *P. spadiceus*

Effect of Moisture and Temperature Level

Maximum production of exoglucanase was observed (Fig. 3) at 70% of moisture level and 30°C of temperature (69.47 IU/mL/min). Increase moisture level from 30 to 70% showed that increase in production of exoglucanasefrom fungus growth. Increase in temperature, initially increased the activity of enzyme because the higher movement of molecules that increase in kinetic energy of exoglucanase (Iram *et al.*, 2021) . As result, the rate of reaction was enhanced, and substrate-enzyme interaction increased. Further increase in temperature caused decrease enzyme activity due to denaturation of structure of proteins, enzyme (Mahmood *et al.*, 2013).

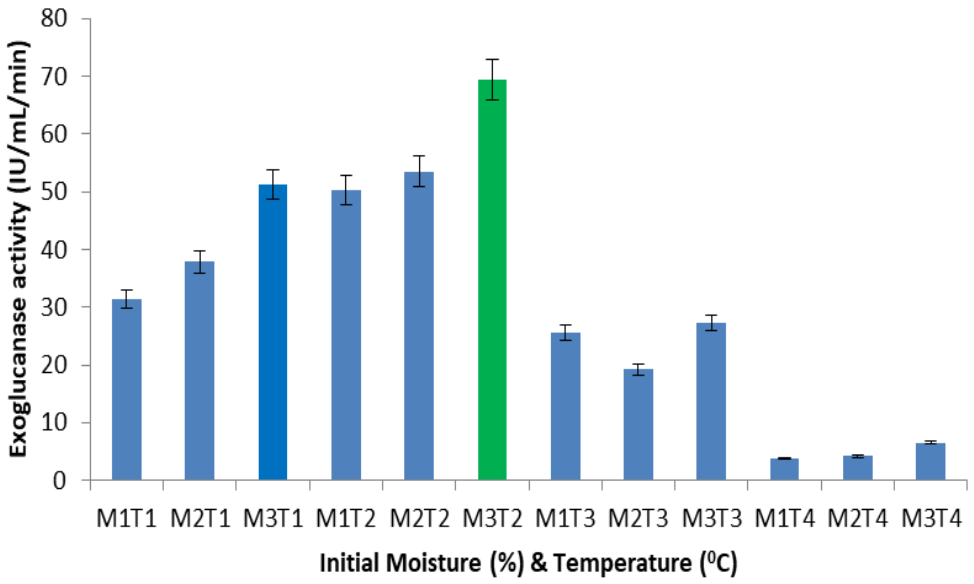

Figure 3. Effect of Moisture and Temperature Level for exoglucanase by *P. spadiceus*

Effect of Carbon and Nitrogen Sources

For carbon source used glucose, fructose and sucrose and for nitrogen source used urea, peptone and ammonium nitrate as additional components for the growth of *P. spadiceus*. Both are used for fungal growth as nutrients sources, as well as exoglucanase production. As carbon source used glucose and nitrogen source used peptone, maximum production was observed (75.8 IU/mL/min), to produce exoglucanase (Fig. 4). External carbon sources (glucose) increase the growth of fungi and production of cellulases because of easily available to fungus than substrate (Gaind and Nain, 2007 and Mahmood *et al.*, 2013). Peptone is better nitrogen source as compared to urea and ammonium nitrate. Sherief et al. also described that the cellulases production enhances with peptone more than urea because amino acidscontain in peptone, readily available as nitrogen source for the growth of A. *fumigatus*and production of enzymes (Trecthewey *et al.*, 2005 and Mahmood *et al*., 2013).

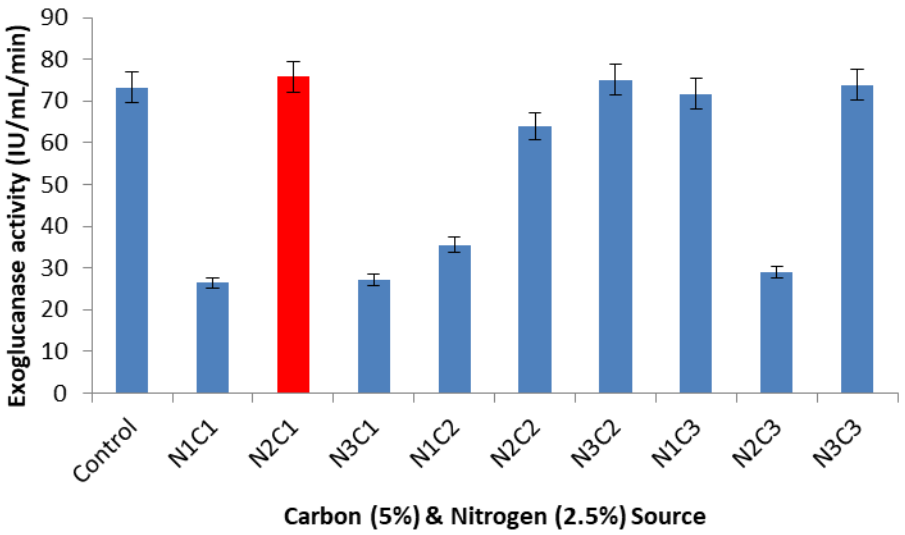

Figure 4. Effect of Carbon and Nitrogen sources on exoglucanase activity

Characterization of Exoglucanase

Optimization of pH for Exoglucanase

For optimum pH for exoglucanase, enzyme assay held under different pH values of sodium citrate buffer (3.5, 4.5 and 5.5) and phosphate buffer (6.5 and 7.5). The maximum activity (53.11 IU/mL/min) was observed at pH 4.5 of sodium citrate buffer. Future increase of pH values of different buffer (Sodium citrate and phosphate buffer),the enzyme activity was decreased probablybecause ionic strength in reaction mixture changed in that case the protein of enzyme is unstable (Fig. 5).

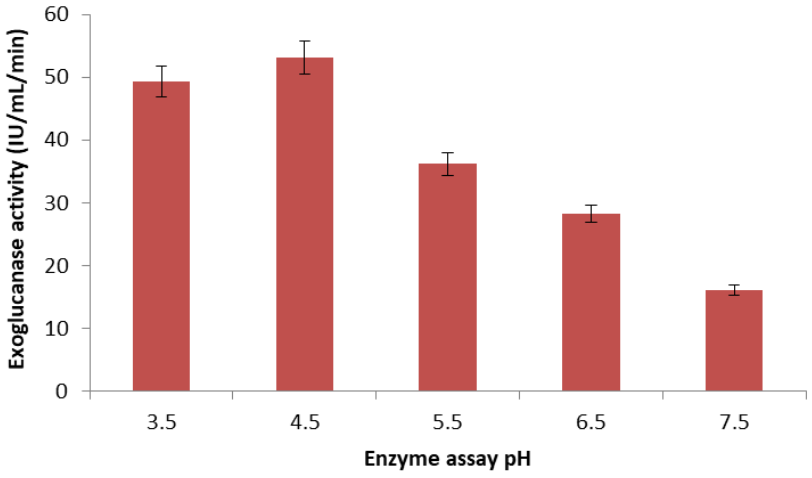

Fig 5: Optimization of pH of exoglucanase

1.1. Optimization of Temperature for Exoglucanase

For optimization of temperature, exoglucanase activity assay was held under at different temperature ranging from 20ºC, 25ºC, 28ºC, 30ºC, 35ºC and 40ºC. In each case,pH was retained at 4.5. Exoglucanase showed maximum activity (64.79 IU/mL/min) at 30°C (Fig. 6). After that, further increased temperature was decreased exoglucanase activity due to denaturation of enzyme structure.

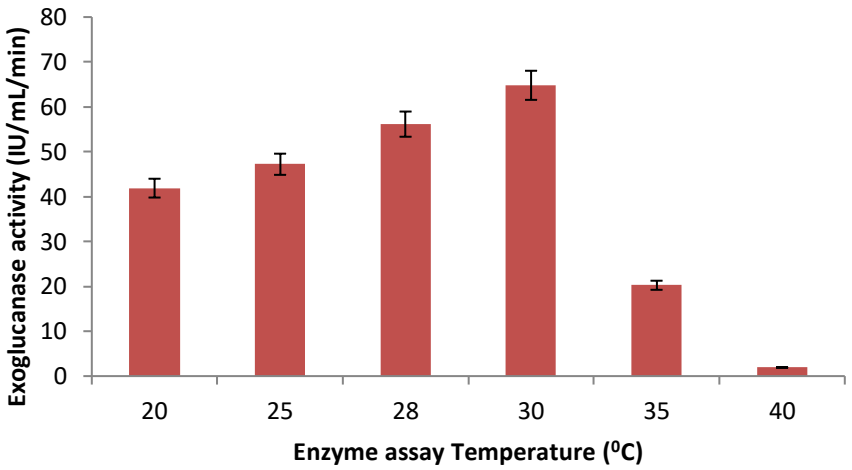

Figure 6. Optimization of temperature of exoglucanase

Optimization of Metal Ions for Exoglucanase

For the optimization of exoglucanase,the different metal ions like Ca^{+2}, K^{+}, Zn^{+2}, Na^{+}, and Mn^{+2} at different concentrations of $CaCl_2$, KCl, $ZnCl_2$, NaCl and $MnCl_2$ were used. As results described that maximum metal ions have positive effects on exoglucanase activity (Fig. 7). Maximum effect on exoglucanase activity was Zn^{+2} ion and KCl, $ZnCl_2$, NaCl and $MnCl_2$ have positive effect on enzyme. These ions increase the production of exoglucanasebecause activating many processes in the fungus like synthesis of proteins and act as a cofactor of enzymes.

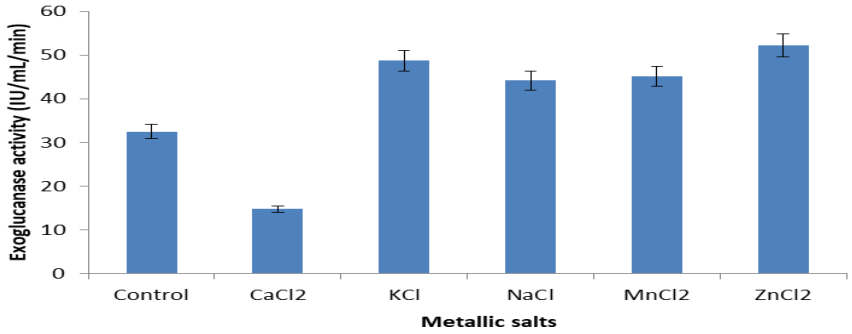

Figure 7. Effect of metal ions on exoglucanase activity

Effect of Substrate on Exoglucanase: Determination of Km and Vmax

The Michaelis–Menten constants Km and Vmaxfor exoglucanase was performed at varying concentration of Avicel (2, 4, 6, 8, and 10 mM) and that constants were measured by Lineweaver–Burk reciprocal plot. The Lineweaver–Burk reciprocal plot between $1/V_0$ (cellulase activity) on Y-axis against 1/[S] (concentration of substrate) on X-axis (Fig. 8) and generated a hyperbolic curve. The values of Km and Vmax for exoglucanase from *P spadiceus*was measured by the Linear equation from the plot to be 34.48 mM and 175.43 μM/ml/min, respectively. Dashtban et al. was stated from *Trichodermareesei*, the value of Km 3.8 mM of exoglucanase (Dashtban et al., 2009 and Mahmood et al., 2013). Ayman et al. was described the Vmax of 1.80 U/mL for exoglucanase by using as a substrate Avicel (Dabaet al., 2011 and Mahmood et al., 2013). Km value of 4.34 mM and Vmax of 7.29 μM/mL of exoglucanase from *Aspergillusfumigatus* was reported by Mahmood et al. (Mahmood et al., 2013).

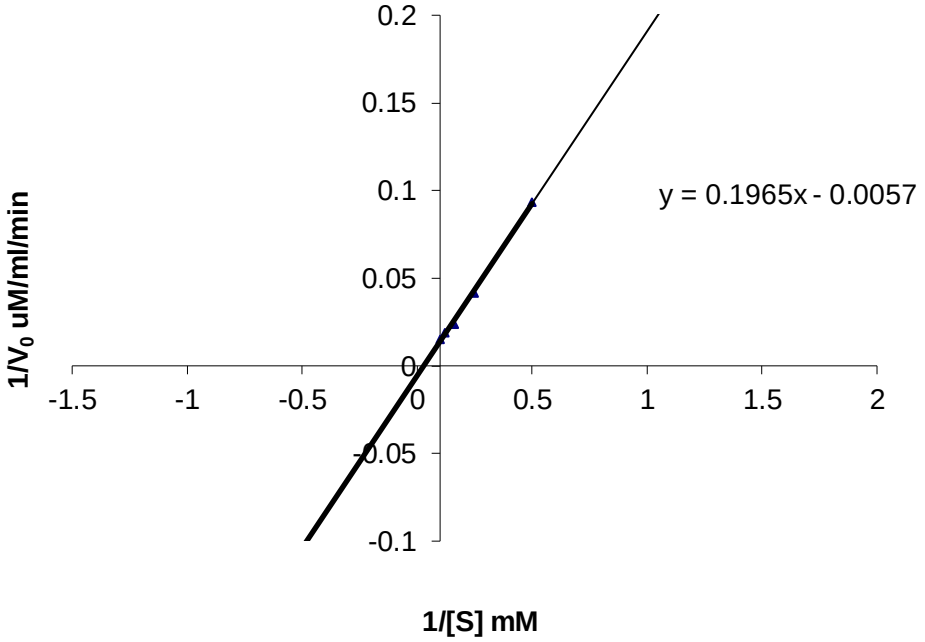

Figure 8. Line-Weaver Burk doubles reciprocal Plot to calculate K_m and V_{max}.

CONCLUSIONS

Exoglucanase takes part in the breakdown of cellulose along with endoglucanase and betaglucosidase. It hydrolyzed oligosaccharides produce by endoglucanase into tri- and disaccharides, called cellobiose. The Solid-state fermentation of wheat straw is producing large amount of exoglucanase by *Phaeolusspadiceus* under improved conditions. Maximum exoglucanase activity was observed at 30°C temperature, day-3 and pH 4.5. Addition of glucose as a carbon source 5% and peptoneas a nitrogen source 2.5% further enhanced the production of exoglucanase. Values of Michaelis–Menten kinetics constants (Km and Vmax) indicated that exoglucanase has high affinity for its substrate (Avicel). Different inorganic metal ions like K^+, Mg^{2+}, and Ca^{2+} and Na^+ have positive effect on enzyme activity but Zn^{2+} have more positive impact on enzyme activity. These ions act as cofactor for exoglucanase or help in the synthesis of proteins. Purification of chromatography techniques, utilization for biofuel production and application at pilot scale can be used for future perspectives.

Conflict of interest: The authors declare no conflict of interest.

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Volume 1, Issue 2. August, 2021. www.jbbt.org

CHEMICAL ANALYSIS AND BIOACTIVITIES OF ESSENTIAL OILS OF SELECTED *MENTHA SPECIES* FROM LOWER HIMALAYA REGION OF PAKISTAN

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ABSTRACT

Plants secondary metabolites are responsible for various biological activities. Essential oils of plants are also secondary metabolites that have curative and pharmaceutical effects such as antibacterial, antifungal, antiviral and insect repellent. The *mentha* species are potential candidates for development of drugs due to their efficient antimicrobial, antioxidant and radical-scavenging activities and reliable cytotoxicity

In the current study *Mentha species* (*Mentha arvensis, M. longifolia* and *M. spicata)* were assessed for their phytochemicals. The essential oils contents by using gas chromatography mass spectroscopy (GC-MS) and FT-IR . The antioxidant, antibacterial, cytotoxic and mosquito repellent activities of plant extracts were also determined It was observed that various mentha extracts are good source of flavonoids, phenolic, tannins and saponins . All mentha specie contained higher quantity of essential oils. The higher antioxidant activity of DPPH assay was found as compared to ABTS and H2O2. Where as higher zone of inhibition was obtained for S aures as compared to other microorganism . Where as plant extracts have shown lower cytotoxicity . It is expected that due to out come of this study raw materials of mentha spp will get industrial application and might be used for preparation of drugs required for human health

Keywords; Phytochemicals , Chemical analysis, Bioactivities**,**

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INTRODUCTION

About 80% of world rural populations rely on herbal remedies for the treatment of various human ailments. Medicinal plants have got the vital role in allopathic drugs, large number of the modern drugs such as digoxin, morphine, codeine, aspirin, vinblastine, cocaine, emetine, ephedrine, vinocristine, pilocarpine etc., have been isolated from plants (Abbasi *et al*., 2015).. After the more advancement of synthetic chemistry during the later part of 20th century over natural products, again the interest of pharmaceutical industry in medicinal plants reawaken and they look at source of herbal plants as chemical scaffolds for synthesis of drugs. The natural compounds from medicinal plants are safer and could be found to replace the synthetic drugs which constitutes about 70% of our drugs (Adedapo *et al.*, 2009).

It was estimated by WHO that world market for drugs originated from plants, may account about Rs. 200 million. Among 2,50,000 plant species that belong to higher plant on land, more than 80,000 are playing a vital role in health care. The drugs were obtained by whole plant or from single part such as stem, leaves, flower, root, bark, seed, etc. Some were derived from excretory product such as resins, latex and gum. Allopathic medicines have adopted a large number of plant derived drugs. These constitutes are an important part of the modern pharmacopoeia (Ashafa *et al.*, 2010). Some plants also contained the important chemical intermediates which are very essential for the manufacturing of modern drugs (solasodine, diosgenin and betaionine) (Agor and Ngogan, 2005).

Pharmaceuticals available in modern medicine are directly or indirectly derived from natural sources, which are also of great importance in the process of drug discovery due to their huge

diversity in nature that permits the identification of lead molecules of much interest for the development of new therapeutic agents for pathological processes. It is believed that about 80% of world's total population use plants as a fundamental source of medicine (Beauchamp and Fridovic. 1971; Cefarelli *et al*., 2006). Modern science and technology plays an important role in approaching for cultivation, conservation and preservation of the herbal drug species through plants tissue culturing and plant molecular biology. The isolation of active constituents and their development into new therapeutics; standardization and other related aspects need to be focused for further development(Dillard and German, 200). The plants of the genus Phyllanthus (Euphorbiaceae) are widely distributed in most tropical and subtropical countries, and have long been used in folk medicine to treat kidney and urinary bladder disturbances, intestinal infections, diabetes, and hepatitis B (Dufour *et al*., 2000).

In the past three decades researchers on plants have revealed that plants have such constituents which have been used for cure and prevention of diseases (Farnsworth and Morris (1976). Plants have molecules which have potential for new drugs discovery and for the treatment of diseases (Feng *et al.*, 2011).

Infectious diseases caused by bacteria are also cured by most of the medicinal plants. These plants are locally available, inexpensive and becoming more popular day by day.Metabolic syndrome causes incompatibilities in the body. Medicinal plants having large excess of phytochemicals were used for treatment of metabolic syndrome. An antibacterial activity have been observed from carbazole alkaloid "clausenol" purified from an alcoholic extract of the stem bark of clausena anisata. The alcoholic and acetonic extract has shown significant in-vitro antibacterial activity from the leaves of Cassia alata (Foster and Duke, 2000). Cytotoxic activity had been shown by plants such as Thevetia ahouai, Physalis viscosa, Piper jacquemontianum and Piper barbatum., Senna occidentalis, Tovomita longifolia and Lippia cardiostegia., Blepharocalyx salicifolius and Senna occidentalis against one cell line, SF-268 or MCF-7, respectively at GI_{50} values $\leq$10 μg/ml (Fowler, 2006) . Cancer is responsible for many deaths worldwide. For treatment of cancer, methanolic extracts of Croton membranaceus roots and Zanthoxylum xanthoxyloides bark showed markedly anti-proliferative activities against three human cancer cell lines such as DLD-1, MCF-7 and M14, using the MTT assay .Anti HIV 1, anti-malarial and anti-fungal activity *in-vitro* was shown by four compounds those were

purified from an extract prepared from the fruit rind of Terminalia belerica viz termilignan, thannilignan, 7-hydroxy 3, 4 (Methylenedioxy) flavone and anolignan B (Graf *et al*., 2010). **The** antimalarial activities were tested in species like Japonica, *Artemisia maritimia* and *Artemisia nilegarica* in their ethanolic and petroleum extracts both *in vivo* and *in vitro*. The activities were conceded out *in vivo* by using Rane test in Balb/c which showed that all composites extended the endurance time of mice .Plants are equipped with many chemical constituents. These chemical constituents are classified into two types of metabolites i-e primary and secondary metabolites (Gulfraz *et al*., 2008) .

Primary metabolites are involved in the primary metabolism of plants. Some of the primary metabolites are nucleic acids, carbohydrates, lipids, proteins and chlorophyll. Plants store the excess of primary metabolites are found either in stem, leaves or roots, which are used as food in our daily life (Hussain *et al*., 2014) .
Secondary metabolites include chemical constituents which do not get metabolized in primary metabolism. In the past they were regarded as excretory products or end products. Now their importance has been realized by the medicine. These constituents are now regarded as medicinally important constituents. Secondary metabolites play an important role of defense for the plants. They protect the plant from insects and also keep the herbivores away from them (Koehn and Carter, 2005).

The plant secondary metabolites could be classified into some major classes such as alkaloids, terpenoids and phenolics etc.Alkaloids are heterocyclic nitrogen containing compounds. They contain nitrogen, they are basic in nature and are alkali like compounds and have bitter taste. These heterocyclics are pharmacologically active compounds. On the basis of number of nitrogen present in the molecule of alkaloids, they are further divided into different structures like Atropine, containing single nitrogen and Ergotamine, containing penta nitogen **.**Alkaloids are crystalline in nature and contain oxygen e.g. Nicotine. Normally alkaloids are colorless but inn few cases they are colored for example serpentine, it is brownish red in color. Alkaloids are soluble in ether, chloroform and other non-polar solvents and divided into three classes (Krymow, 2002).

Terpenoids are used for medicinal purposes as well as for dietary purposes Terpenoids are used to flavor the food and drinks. These are also used as flavoring agents in toothpastes and cigarettes (Males and Farnsworth,1995). Wound healing and anti-microbial activities of terpenoids are reported. These types of terpenoids are present in the resins obtained from the plants. Phenolics are made up of hydroxyl group containing aromatic ring. There may be one or more hydroxyl group .Tannins, lignin polyphenolics and melanin are the polymeric phenolic compounds of plants which are medicinally important. In phenolic compounds, mostly sugar moieties are present therefore they are soluble in water (Gulfraz *et al*., 2008). In human diet

flavonoids and polyphenolic compounds are commonly found and these are class of secondary metabolites. Tannins and Saponins are also a type of flavonoids. There is a close relationship between medicinal plants and drug discovery. Use of medicinal plants for treatment of diseases by humans has long history. Isolation of active compounds from medicinal plants led to the foundation of drug discovery. Morphine was isolated in 1817 by Serturner (pharmacist) from Opium. Isolation of caffeine, atropine, codeine was carried out between 1820-1850. German scientist discovered first synthetic drug and was named as Antipyrin (Newman and Cragg,2012). Pakistan has been bestowed with unlimited natural resources of medicinal plants. In Pakistan there are 25 medicine companies producing tibbi medicines on commercial scale. Export Promotion Bureau of Pakistan reported that in 1999, Pakistan exported 8500 tons of medicinal plant materials and yet earned 6 million US dollars as compared to 31 million dollars spent on import of herbal raw materials from other countries (Newman *et al*., 2000; Rios, 2010) .Almost 50,000 herbalists are working in different areas of Pakistan. 2000 species of Pakistani plants are given position in Unani system but only 400-600 have been documented. Pakistan pharmaceutical industries depends on medicinal plants for production of drugs and local practitioners uses these medicinal plants for cure of human ailments (Packer *et al*., 2004). Similarly 70 species of medicinal plants (Pandey,2006). In Pakistan, a large variety of lamiaceae flora is inhabited consisting of total 91 species of family lamiacea which include 6 Mentha. The Northern part of the country is an important hotspot of the floral diversity and has a collection of many important indigenous medicinal plants that are regularly used by the people living in the surrounding area. Ethnomedicinally, the plant species of lamiaceae which are used by local people of the area includes *Mentha arvensis* and *Mentha piperita*, commonly known as podina, is used as emollient, operient, demulcent, antidiabetic and carminative while *Oreganum vulgare* (Ban ajwain) is used for colic, uterine disorders and epilepsy. *Mentha longifolia* is used as a condiment, carminative and stimulant, remedy for headaches, stomach trouble, digestion, purification of blood and anti-emetic. *Salvia nubicola* is used in treatment of lung diseases and asthma of cattles (Phillipson,1994). Keeping in view impotence of medicinal plants especially *menthe longifolia*, present study was under taken with following aims and objective Extraction of essential oils from plants and chemical analysis and quantification of constituents of essential oils by gas chromatography-mass

spectrometry Exploring the anti-microbial activity of essential oils against human pathogenic bacteria.

MATERIAL AND METHODS

Collection of samples

Fresh aerial parts of *Mentha arvensis, Mentha longifolia* and *Mentha spicata* were collected from District Abbottabad. The samples were collected on the basis of ethnobotanical uses of menthe species in folk medicines by inhabitants of this area. The samples were collected in fine plastic bags duly labeled with name, location and area of collection of plant samples. The samples were identified by expert taxonomist at the Department of Environmental Sciences, COMSATS Institute of Information Technology, Abbottabad and voucher specimens (No. 132) were deposited for future reference. About 3 kg of hand plucked plant materials were packed in plastic bags and shifted to Biochemistry laboratory, Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad. The aerial parts of the *mentha* species were utilized for the extraction of essential oils as well as for determination of other phytochemicals and for assessment of bioactivities.


Preparation of samples

Plant materials were washed with distilled water to remove unwanted materials including dust. Then samples were shade dried followed by sun and oven drying at lower temperature for one night. The dried samples were ground with the help of electric grinder, sieve 80 mesh and stored at 4 ℃ till further use.

Analysis of plant extracts for phytochemicals

Determination of flavonoids

The total flavonoid content (TFC) was determined by spectrophotometric method (Skehan *et al*., 1990), with some modifications and analysis were carried out in triplicate form. A 100 ppm standard solution of Quercetin was prepared in conical flask by adding 2.5 mg of Quercetin in 25 mL methanol. The TFC of sample was determined by using standard curve made by using six different concentrations (10, 20, 40, 60,80 and 100 ug/mL) of (Sigma-Aldrich). Sample solution (0.5mL) was put in all falcon tubes to which 0.5 mL distilled water and then 0.5 ml solution of $AlCl_3.6H_2O$ was added. Then the falcon tubes were incubated for 5 mins at room temperature. After incubation, 2mL of NaOH solution (1M) was added and mixed well. The absorbance was measured at 510 nm by using spectrophotometer. The total flavonoids were expressed as µg of quercetin equivalent (QE) per g of sample. Three replicates of each sample was used and average results were calculated as QEg/100g.

Estimation of Total Phenolic Content

Total phenol content (TPC) in plant extracts was generally determined according to Folin-ciocalteu method (Phillipson, 1994). A 100 ppm standard solution of Gallic acid was prepared in methanol (2.5 mg of gallic acid in 25mL methanol) in a conical flask. The TPC of sample was determined by standard curve made by using different concentrations (10, 20, 40, 60, 80 and 100 µg/mL) of gallic acid (Sigma-Aldrich). 0.5 mL sample solution and 0.5 ml distilled water was mixed with 1ml of 10 % Folin-Ciocalteu's phenol reagent. After incubation for 5 mins at room temperature, 2mL of sodium carbonate solution was added and was then mixed well. Then the mixture was incubated at room temperature in dark for 90 mins. After incubation, the absorbance of solution was recorded at 760 nm versus the blank on UV-Visible spectrophotometer. Three replicates of each sample were taken and final results were expressed as standard Gallic acid equivalent (Gulfraz *et al*., 2008).

Determination of Saponins

Saponin content from three plant samples was determined according to the method described by (Skehan *et al.*,1990). The 20g of each plant sample was dispersed in 200 mL of 20% ethanol. The suspension was heated over a hot water bath for 4 hrs with continuous stirring at about 55 °C. The mixture was filtered and the residue was re-extracted with another 200 mL of 20% ethanol. The combined extracts were reduced to 40mL over water bath at about 90 °C. The concentrate was transferred into a 250 mL separatory funnel and 20 mL of the diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ethyl ether was discarded. The purification process was repeated and 60 mL of n-butanol was added. The combined n-butanol extracts were washed twice with 10 mL of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight and saponin content was calculated in percentage (Schwartmann *et al*., 2002; Sharma *et al*., 1975).

Quantification of tannins

Extraction of tannins was carrie out according to the method reported by (Ullah and Khan, 2008) . A mass of 100g of dried powder of plant was placed in an Erlenmeyer flask with 400mL of 70% acetone. The flask was covered with aluminium foil to avoid light exposure. This mixture was refluxed at 60 °C for 12 hrs. After this process, the sample was filtered using Whatman filter paper no.41 and centrifuged at 3500 rpm for 15 mins. The solvent was removed using a rotary evaporator at a maintained temperature below 60 °C and light exposure was avoided by wrapping the flask in aluminium foil. Quantitative determination of total tannins was carried by Folin-ciocalteu method. In this assay, 800 µL of the sample was put into a test tube and mixed with the same volume of Folin-ciocalteu reagent, shaken and left for 5 mins. The solution was diluted with 5 mL of distilled water and analyzed in a UV-Visible spectrophotometer at 725 nm for the determination of total tannins. The obtained absorbance values were analyzed against the standard curves prepared with tannic acid for total tannins and expressed as mg standard equivalent/g of plant extract.

Extraction of Essential oils

Steam distillation is a separation process used to purify or isolate temperature sensitive materials, like natural aromatic compounds. Steam or water is added to the distillation apparatus to lower the boiling points of the compounds. The basic principle of steam distillation is that it allows a compound or mixture of compounds to be extracted at a temperature considerably below that of the boiling point of the individual constituent. Essential oils contain substances with lower boiling points and in the presence of steam or boiling water, these compounds are volatilized at a temperature of about 100 °C. The essential oil from fresh aerial parts of the plant was extracted. The aerial parts of the selected plants were cut into smaller pieces with the help of knife and scissors. The small pieces of plant material could yield greater biomass that is easily extracted with high efficiency. After converting into small pieces, the weight of aerial parts of plant was determined by using an electric digital balance. The weighed plant material was then charged into a stain-less vessel of distillation apparatus. Approximately 2000 mL distilled water was added to the vessel and then placed on heating mantle. The vessel was then connected with a condenser that was cooled by cold tap water. The flow of water through a condenser was started and then the resulting mixture was boiled for 3 hrs. The distillate containing volatile compounds was collected in a separating funnel after passing through the water condenser. Layer of oil at the top of water in separating flask clearly revealed the presence of essential oil. Then essential oil floating on distillate was separated and the remaining distillate was used to separate oil from it by liquid-liquid extraction (Upadhyay, 2015).

Liquid-Liquid extraction

Liquid-liquid extraction (solvent extraction) is a separation process which is based on the different distribution of the components to be separated between two liquid phases. As essential oil is a mixture of organic compounds so easily extracted by using organic solvents like hexane, chloroform, ethyl acetate, methanol or acetone etc. But in liquid-liquid extraction from water substrate, only water immiscible solvents could be used which include hexane, ethyl acetate and chloroform etc. In this process 70 mL HPLC grade n-hexane was added to distillate collected in separating funnel. The stopper of separating funnel was opened just after the addition of hexane to reduce the pressure produced due to hydration of hexane. The stopper of funnel was closed

and the resulting mixture was shaken gently for some time to dissolve maximum amount of essential oil in hexane. The stopper of separating funnel was opened for some time to release pressure. The shaking process was repeated at least three times and the separating funnel was hanged in a stand holder. After a few minutes, a clear layer of hexane containing essential oil was formed above the water which was separated in another flask. The same procedure was repeated three times by adding 70 mL of hexane in the residual distillates. Some traces of water still present in hexane extract was removed by the addition of small amount of anhydrous magnesium sulphate to hexane extract. Hexane extract containing magnesium sulphate was then filtered in a pre-weighed round bottom flask (Valko *et al.*,2006).

The excess hexane was evaporated by the help of rotary evaporator (Buchi R100) at 25°C under reduced pressure. The hexane free essential oil was weighed on an analytical balance and the yield (%) of the oil was determined by dividing the extracted oil mass by the mass of plant used for the extraction of oil. Round bottom flask containing hexane extract was connected to rotary evaporator by means of specific glass adopter. A collecting flask was connected to the condenser of rotary evaporator to collect the hexane after evaporation. At the start, the vacuum of rotary evaporator was set at 200 mbar at 25 °C. After 3 mins, when the evaporation from flask stabilized, the pressure was reduced to 100 or 80 mbar. This process was carried out until all the hexane was evaporated from round bottom flask. The pure essential oil was weighed and the yield (%) of the essential oil was determined by dividing the mass of essential oil to the mass of fresh plant material used for the extraction of oil.

Chemical analysis of the essential oil by GC-MS

The chemical composition of the extracted oil was investigated by using Gas Chromatography-Mass Spectrometry (GC-MS). The Hawlett Packard GC-MS system was used to analyze the samples. The Hawlett Packard GC 6890N was equipped with DB-5 column (30 m length, 0.25 mm internal diameter and 0.25 µm stationary phase film thickness) and coupled with a HP 5973 Mass Spectrometer (MS; Agilent Technologies Inc. USA). The injector was operated at 235C . The oven temperature of GC was programmed as: the initial temperature of oven was 40 °C and it was maintained for 2 min, the temperature was raised from 40 to 450 °C at a heating rate of 4 °C and remained at higher temperature for 8 mins. Highly pure helium gas was used as mobile

phase with a constant flow of 1mL/min. The dilute solution of essential oil was injected with volume of 1 µL in split less mode (Upadhyay, 2015).

The FT-IR spectra of defatted samples was recorded in FT-IR instrument (Model 1:1 FS 25, Bruchure, Germany) with PC based software controlled instrument operation and data processing. A small amount of powdered sample made in pellets using KBr for FT-IR analysis and a thin film was prepared by applying pressure. The data of IR transmittance was collected at a wave number ranged from 4000 cm^{-1} to 500 cm^{-1}. All the samples were analysed triplicates with KBr pellets as blank. The spectra were compared with a reference to identify the functional groups existing in samples.

Determination of bioactivities

The antioxidant activity of plant extracts was carried out according to method earlier reported by many authors . Therefore for determination of antioxidants, following bioassays were used.

DPPH scavenging activity was done according to method (Graf *et al*., 2010) with some modifications. This method was based on the ability of antioxidant to scavenge *1, 1- diphenyl-2-picryl hydrazyl* (DPPH) action radical. In a falcon tube, 100 µL of the sample solution was taken and then 4 mL of DPPH solution (0.1 mM) was added and mixture was vortexed vigorously. Then the mixture was incubated with Aluminium foil to avoid heat exposure in the dark for 30 mins at room temperature. *UV- Vis spectrophotometer was used to measure the absorbance of the solution at 517 nm.* Percentage inhibition was calculated by

$$\% \text{ Inhibition} = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

ABTS scavenging bioassay

The ABTS radical scavenging bioassay was carried out. The ABTS radical cation was prepared by mixing 5 mL of a 7 mM aqueous ABTS solution to about 88µL of 140 mM potassium persulphate ($K_2S_2O_8$) by making the final concentration of a solution to be 2.45 mM. The solution was incubated in dark for 16 hours. After incubation, the radical cation was further diluted in ethanol just to make the initial absorbance value of solution to be 0.7. Then the plant extracts to be studied were prepared in ethanol to a volume equal to 25µL. 10µL of sample solution was added to the reaction mixture and the final percentage reduction in absorbance was

measured at 730 nm. The expected final absorbance was assumed to be 20-80% decreased as compared to the initial absorbance of reaction mixture (Wall *et al*., 1966).

Scavenging of H_2O_2

Scavenging of H_2O_2 was determined. About 4 mM H_2O_2 0.6mL solution (prepared in PBS) was added to 4mL solution of extract and incubated for 10 min. The absorbance of the solution was measured at 230 nm against a blank solution, by using spectrophotometer method. Free radical scavenging potency as determined from %age H_2O_2. Lower H_2O_2 indicated strong free radical scavenging activity (Ullah and Khan,2008).

Brine Shrimp toxicity assay

Brine shrimp eggs were hatched in a shallow rectangular dish (22 × 30 cm) filled with artificial sea water which was prepared with a commercial salt mixture and double distilled water. A plastic divider with 2 mm holes was clamped in the dish to make two unequal compartments, the eggs (50 mg) were sprinkled into the larger compartment which was darkened while the smaller compartment was illuminated. After 48 hrs, the phototropic napulii were collected by pipette from the illuminated side, which was separated by the divider from their shells. Twenty shrimps were transferred to each sample vial using pipette and 5 mL artificial seawater was added. The napulii can be counted in the stem of pipette against a lighted background. A drop of dry yeast suspension (3 mg in 6 mL artificial seawater) was added as food to each vial. The vials were maintained under illumination. Survivors were counted with the aid of 3 magnifying glasss and after 24 hrs percent death at each dose and control were determined. In each case where control deaths occurred, the data were corrected using Abbott's formula:

$$\% \text{ death} = [\text{Test} - \text{Control}] / \text{Control} \times 100$$

Determination of Antibacterial activity

Suspension of organisms was prepared as per McFarland's standard. A 24 hours old culture was used for the preparation of bacterial suspension. Bacterial strains (colony) were picked in eppendorf tubes with the help of *inoculation loops* and placed in 37°C incubator for 30 minutes. Each bacterial colony collected was then mixed in 3 mL distilled water and then shaked vigorously or vortexed and optical density was checked and made equal to 0.5 by using spectrophotometer at 600 nm wavelength.

Antibacterial activity of mentha essential oil

Antibacterial activity of essential oils extracted from M. arvensis, M. longifolia and M.spicata was assessed using agar well diffusion method (Upadhyay, 2015) . Nutrient agar was prepared by pouring accurately weighed 13g/L of Nutrient broth and 14 g/L of agar technical in 1L of distilled water in reagent bottle and was mixed thoroughly so that it may get mixed. Then the mixture was autoclaved *with the temperature at 110 °C for 60 mins. Then* agar plates were poured by 30-35 mLof autoclaved nutrient media, covered and sealed with parafilm. The *covered plates were allowed to rest for an hour so the agar can solidify* and then petri plates were placed in an incubator at 37^{o} C for overnight. 200 µL of each bacterial strain was evenly spread on surface of petri plates using glass spreader. Then four uniform and equidistant wells were made with 6 mm (diameter) cork borer in each plate. Each well was filled with 30µL of essential oil test solution and 2 replicates of each test petri plate were made. Hexane was used as negative control and streptomycin (1 mg / ml) was selected for positive control as the reference for all bacterial strains. After half an hour, the plates were placed in incubator at 37°C for 24 hours. The size of zone of inhibition of each well in a petri plate was individually measured in millimeters *by using a scale at four different places around the inhibition zone circle* (Wall et al., 1966).

The biological activity tests were conducted by using different concentration ranging from 1-5%. For the biological activities the essential oils solutions were prepared in absolute ethanol. For the preparation of 5% solution, about 1mL of ethanol was added for each 50 mg of essential oil (50 mg/mL). The same procedure was continued for 1% solution in ethanol (10 mg/mL).

Statistical analysis

The bioassay results are presented as an average of four to five replicates data. In way of finding the statistical difference between different treatments, the data obtained were analyzed by using one way ANOVA (analysis of variance).

RESULTS

Analysis of Phytochemicals

Quantitative analysis of extracts indicates the percentage of flavonoids, phenolics, saponins and tanins (Table 1). Result indicated that the aerial parts of *M. spicata* contained the higher amount of flavonoids (15.05 mg/g) followed by *M. longifolia* which contain 14.17mg/g of flavonoids while M. arvensis has 12.21mg/g of flavonoid content in plant extracts. The percentage mg per gram of sample extract of total phenolics was 20.32, 25.149 and 27.637 in *M. arvensis, M. longifoilia* and *M. spicata* respectively. The amount of saponins was maximum in plant extract of M. spicata (0.134 mg/g) whereas *M. arvensis* and *M. longifolia* contained 0.04 and 0.01 mg/g of saponins. Similarly the yield obtained of tannins was significant in *M. spicata* (3.51 mg/g) followed by 3.14 mg/g in *M. longifolia* extract while 2.31 mg/g of tannins was found in extract of *M. arvensis*.

Table 1. Quantitative analysis of phytochemicals

Plant name	Flavonoids (mg/g)	Total phenolics (mg/g)	Saponins (mg/g)	Tannin (mg/g)
M. arvensis	12.21±0.07	20.32±0.09	0.040±1.3	2.31±1.5
M. longifolia	14.17±0.07	25.149±0.06	0.013±1.2	3.14±0.9
M. spicata	15.05±0.14	27.637±0.06	0.134±1.2	3.51±0.8

Mean ± Standard Deviation (n=3)

Assessment of yield of essential oils

Essential oils were extracted by hydro distillation from fresh aerial parts of *Mentha arvensis, Mentha longifolia and Mentha spicata.* Fresh aerial parts of *M. arvensis* and *M. longifolia* were rich in essential oil and yielded 1.70% and 1.47% essential oil respectively. M. spicata also showed good yield however, the amount was less as compared with other mentha species (Table 2).

Table 2. Percentage yield of essential oils

Latin name	Family	%age yield of essential oils
Mentha arvensis	Lamiaceae	1.70±0.11
Mentha longifolia	Lamiaceae	1.47±0.31

Mentha spicata	Lamiaceae	1.20±0.03

Mean ± Standard Deviation (n=3)

Chemical analysis of essential oils of *mentha* with GC-MS

The chemical constituents of *mentha* oils were obtained by analysis of oil by GC-MS and results are presented in Tables 3 and 4. In the essential oil of *M. arvensis*, seven main compounds were identified representing (99.1%) of the essential oil. The most abundant compounds were carvone (84.3%) and eucalyptol(5.3%). *M. longifolia* essential oil contained six major compounds representing 98.2% of the oil. Major compounds were piperitone oxide (54.2%), menthone (9.7%), 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (8.4%)**,** borneol (4.6%), pulegone (4%) and menthol (3.3%). In *M. spicata* essential oil, four major compounds were identified constituting 96.5% of the oil. The major compounds were 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (47.1%), eucalyptol (12.0%), α-pinene (9.7%) and borneol (9.5%).

Table 3. Chemical composition of *M. arvensis* essential oil based on total ion chromatogram of GC-MS

S. No	Compound name	RI	RT	%age
1	α-Pinene	930	10.81	0.1
2	Sabinene	970	12.23	0.1
3	β-Pinene	973	12.34	0.1
4	β-Myrcene	988	12.86	0.1
5	3-Octanol	991	12.99	0.1
6	Limonene	1026	14.25	3.2
7	1,8-Cineol	1028	14.33	5.3
8	β-cis-Ocimene	1035	14.58	0.1
9	Cis-Sabinene hydrate	1064	15.66	0.1
10	Linalool	1096	16.86	0.3
11	Trans-p-mentha-2,8-dienol	1118	17.63	0.1
12	Trans-Limonene oxide	1135	18.27	0.1
13	Borneol	1164	19.33	0.7
14	4-Terpineol	1175	19.73	0.1
15	α-Terpineol	1188	20.21	0.4
16	Dihydrocarveol	1193	20.38	0.1

17	Cis-Dihydrocarvone	1195	20.43	0.4
18	Cis-Carveol	1231	21.69	0.3
19	Carvone	1246	22.22	84.3
20	Isopiperitenone	1269	23.03	0.3
21	Piperitenone	1339	25.39	0.2
22	β-Bourbenone	1386	26.94	0.4
23	Jasmone	1396	27.27	0.5
24	Caryophyllene	1422	28.07	0.6
25	β-Farnesene	1455	29.1	0.1
26	Germacrene-D	1483	29.97	0.7
27	Elixene	1499	30.46	0.1
28	Caryophyllene oxide	1586	33.04	0.1
	Total % Identified compounds			**99.1**
25	Piperitenone	1339	25.39	0.2
26	1-Cyclohexene-1-methanol, 4-(1-methylethenyl)-, acetate	1345	25.57	0.1
27	2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one	1365	26.25	8.4
28	β-Bourbenone	1386	26.94	0.1
29	Caryophyllene	1422	28.07	1.7
30	Germacrene-D	1483	29.97	0.7
31	Caryophyllene oxide	1586	33.04	0.3
	Total % Identified compounds			**98.2**

Chemical Constituents of *mentha* oils

The identified compounds were listed according to their elution order on DB-5 GC column. Carvone (84.3%), piperitone oxide (54.2%) and 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (47.1%) are the three major compounds present in *M. arvensis, M. longifolia* and *M. spicata* respectively. Similarly some compounds were found common in plants. For example, eucalyptol was present in higher concentration in *M. spicata* (12%) followed by (5.3%) in *M. arvensis* while it was lowest (0.4%) in *M. longifolia* (Table 3-5). In the same way the concentration of germacrene-D was (2.5%) in *M. arvensis* followed by (0.7%) in *M. longifolia* and *M.spicata* each. Borneol was (9.5%) in *M. spicata* while (4.6%) in

M. longifolia. Caryophyllene was also common in all the plants though its concentration varied in each plant that is (0.6%), (1.7%) and (1.3%) in *M. arvensis*, *M. longifolia* and *M. spicata* respectively.

Analysis of extracts with FT-IR

Fourier Transform Infrared Spectroscopy (FT-IR) identifies chemical bonds in a molecule producing a profile of the sample, a distinctive molecule fingerprint that can be used to screen and scan samples for many different components. FT-IR is an effective analytical technique for detecting the functional groups and characterizing covalent bonds.

Table 4. FT-IR analysis of methanolic extracts of *mentha* species

Sr. No	Wavelength	Bond	Functional Group
1	3337.45	O-H, H-bond	Alcohol, Phenol
2	2924.51	C-H stretch	Alkanes
3	1710.21	C=O stretch	Carboxylic acids, Carbonyls
4	1514.05	CO asymmetric stretch	Nitro compounds
5	1451.07	C-C stretch	Aromatic
6	1030.75	C-O	Alcohols, Esters, Ethers
7	817.84	C-Cl stretch	Alkyl halides
8	725.27	C-Cl stretch	Alkyl halides
9	632.43	C-Br stretch	Alkyl halides

Antioxidant activities

DPPH and ABTS scavenging potential

For the evaluation of antioxidant activities of plant extracts of M. arvensis, M. longifolia and M. spicata, the DPPH and ABTS scavenging bioassays were employed. The free radical scavenging activity of the

plant extracts was much enhanced in ABTS scavenging assay as compared to DPPH scavenging assay (Tables 5 to 6).

Table 5. Scavenging potential of DPPH

	DPPH Scavenging activity			
Plant extracts Conc. µg/mL	***M. arvensis***	***M. longifolia***	***M. spicata***	***Ascorbic acid***
50	60.7±1.07	70.33±0.4	80.1±0.8	49.29±0.5
100	55.45±1.9	63.77±0.6	73.24±0.62	41.34±1.3
150	48.9±0.13	51.81±0.5	61.33±0.3	33.41±1.7
200	33.62±0.36	36.18±1.0	49.67±0.03	28.67±1.03
250	21.8±0.33	25.42±1.01	33.33±0.2	21.8±1.01
300	19.9±0.33	14.33±2.1	21.9±0.7	13.55±1.23

Table 6. Scavenging potential of ABTS

	ABTS Scavenging activity			
Plant extracts Conc. µg/mL	***M. arvensis***	***M. longifolia***	***M. spicata***	***Ascorbic acid***
50	67.7±1.23	78.73±0.5	88.1±0.9	67.29±0.9
100	59.42±1.8	65.45±0.9	78.44±0.7	56.76±1.09
150	51.89±0.45	58.21±0.7	63.81±0.8	49.87±1.8
200	44.32±0.23	47.81±1.1	55.54±0.01	40.54±1.08

250	38.61±0.10	36.72±1.01	49.01±0.3	34.6±1.1
300	33.62±0.33	29.88±2.1	35.62±0.7	22.55±1.23

H_2O_2 Scavenging potential

The extracts of *M. arvensis*, *M. longifolia* and *M. spicata* significantly scavenged H_2O_2 by 34.9%, 29.66% and 37.9% respectively at concentration of 300 µg/mL (Table 7). *M. arvensis*, *M. longifolia* and *M. spicata* extracts exhibited significant scavenging activity for H_2O_2 on dose dependent manner as shown in table 9.

Table 7 . Scavenging potential of hydrogen peroxide (H_2O_2) by plant extracts

	Percentage of H_2O_2 (%)			
Plant extracts Conc. µg/mL	***M. arvensis***	***M. longifolia***	***M. spicata***	***Ascorbic acid***
50	92.7±1.7	96.46±2.3	87.1±0.6	64.29±0.9
100	90.45±0.9	91.06±0.9	79.05±0.88	52.07±1.2
150	76.9±0.13	83.33±0.77	78.98±0.1	33.41±1.44
200	69.77±0.56	67.11±5.1	67.01±0.05	31.05±1.08
250	51.8±0.45	52.01±1.13	49.34±0.7	24.8±1.1
300	34.9±0.34	29.66±3.22	37.9±0.1	20.45±1.01

Mean±S.D after triplicate analysis

Determination of antibacterial activity

Antibacterial activity of essential oil of *M. arvensis, M. longifolia* and *M. spicata was tested against* the strains of Gram+ bacteria such as *Staphylococcus aureus(KX262674)* and *Bacillus cereus(KX262674*) ;and Gram – bacteria *Escherichia coli (ATCC 10536)*, *Salmonella typhi (ATCC 6539)* and *Pseudomonas aeruginosa (ATCC 9027*). The results indicate that essential oils at different concentrations exhibit significant antibacterial activity. *M. arvensis* showed best antibacterial activity against *S. aureus*, *B. cereus* and *S. typhi* comparable to standard drug used, streptomycine, at concentrations of 100 µg/mL and 200 µg/mL with inhibition zones (9.87, 11.31), (9.36, 11.43) and (6.78, 10.87) respectively in mm. Similarly *M. longifolia* also showed markable antibacterial activity against *B. cereus* with inhibition zones 8.06 mm and 8.25mm at 100µg/mL and 200 µg/mL concentration respectively whereas at conc. 200 µg/mL the inhibition zone of 12.16 mm was measured against *S. typhi*. The essential oil of *M. spicata* showed very good antibacterial activity against *S. aureus* revealing inhibition zones of 8.18 mm and 9.34 mm at 100 µg/mL and 200 µg/mL concentration.

Table 8. Antibacterial activity of essential oils

Sample	Zone of inhibition (mm)				
Essential oil	*P. aeruginosa*	*S. aureus*	*B. cereus*	*E. coli*	*S. typhi*
M. arvensis	2.56±0.23	4.5±0.25	4.43±0.23	2.75±0.20	0.87±0.43
M. longifolia	0	3.25±0.16	3.62±0.32	0	3.12±0.43
M. spicata	3.84±0.31	4.75±0.20	3±0.23	1.75±0.5	0
Streptomycine	9.82±0.43	11.65±0.51	11.07±0.32	9.90±0.25	10.79±0.63
n-hexane	0	0	0	0	0

Table 11. Antibacterial activity of essential oils at

Sample	Zone of inhibition (mm)				
Essential oil	*P. aeruginosa*	*S. aureus*	*B. cereus*	*E. coli*	*S. typhi*
M. arvensis	4.75±0.20	6.25±0.30	6.31±0.47	3.56±0.12	3.37±0.14
M. longifolia	0	5.43±0.23	7.18±0.23	2.18±0.12	5.68±0.12
M. spicata	5±0.88	6.18±0.32	4.62±0.14	2.62±0.25	0
Streptomycine	±	10.18±0.23	13.18±0.37	10.18±0.12	±
n-hexane	0	0	0	0	0

Determination of cytotoxicity

The brine shrimp cytotoxicity of extracts of *M. arvensis, M. longifolia and M. spicata* was carried out which indicates that the cytotoxicity of extracts increased by increasing concentration of plant extracts, however overall extracts has shown less cytotoxicity.

Table 9. Brine shrimp assay of plant extracts

Sample name	Conc. (µg/mL)	Total napuli	% of death after 24 hours	
			Live	Dead
M. arvensis	10	20	18	2
	100	20	15	5
	300	18	11	7
	600	19	10	9

M. longifolia	10	18	17	1
	100	20	14	6
	300	20	12	8
	600	19	10	9
M. spicata	10	20	20	0
	100	19	17	2
	300	18	13	5
	600	18	9	9

DISCUSSION

Estimation of phytochemicals

Phytochemicals present in the plants may be used as food and medicine. There is growing worldwide interest for characterization, isolation, the *in vivo* and *in vitro* assessment of biological activities of the phytochemicals to have beneficial therapeutic capacity for curing human from various illness. Therefore, variety of chemical compounds obtained by the tested plants impart significant anti proliferative, cytotoxic, antibacterial and anti-tuberculosis activities. In the present study, the plant extracts were assessed for the presence of flavonoids, total phenolics, saponin and tannin. The study indicates that the presence of these phytochemicals in all the three plants. *M. spicata* extract contained the considerable amount of flavonoids, phenolics and tannins (Flowler, 2006; Graf *et al*., 2010; Koehn and Carter, 2005). .Flavonoids are polyphenolic molecules containing 15 carbon atoms and are soluble in water. Flavonoids are a group of plants metabolites thought to provide health benefits through cell signaling pathways and antioxidant effects (Newan and Cragg, 2012). Phenols constitute the largest group of secondary metabolites, varying in size from a simple structure with aromatic ring to complex ones. Phenolic compounds, ubiquitous in plants are anssential part of human diet, and are of considerable interest due to their antioxidant properties. Saponins are class of chemical compounds found in various plant species. Furthermore saponins glycosides have many health benefits of Tannins (flavonoids) are astringent polyphenolic biomolecules that binds to and precipitates proteins and various organic compounds including aminoacids and alkaloids (Rios, 2010, Packer *et al*.,2004).

Phytochemical prospection of *M. arvensis* extract indicated the presence of different secondary metabolites (Koehn and Carter, 2005). So the presence of significant amount of these important phyto-constituents bestow the plant with high medicinal activities like free radical scavenging and antimicrobial activities (Marles and Farnsworth, 1995).

Chemical composition of Mentha spp

By comparison of chromatogram of GC-MS, the area of peaks of compounds was calculated and other required information was obtained by NIST library data base. The prominent peaks in the chromatogram of essential oil of *M. arvensis* indicated the presence of carvone (84.3%), eucalyptol (5.3%) and limonene (3.2%) whi le other chemical compounds were present in lower quantities. It was reported in literature that experiment conducted in Brazil for analysis of *M. arvensis* and GC-MS indicated a significant amount of menthone (69.77%) while p-Mentone (12.00%) have shown an excellent antibacterial activity While another reported data indicates the presence of Menthol (78.90%) and isomenthone (6.35%) in the essential oil of M. arvensis (Hussan *et al*., 2014).

The chemical composition of essential oil of M. longifolia contains the major compounds to be piperitone oxide (54.2%), menthone (9.7%), 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (8.4%), Borneol (4.6%), Pulegone(4%), 1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octan-6-one (3.6%) and Caryophyllene (1.7%). Similarly, the GC-MS analysis of chemical compounds in M. spicata revealed that the major compound found in its essential oil was 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (47.1%). 1,8-cineol (12.0%), α-Pinene (9.7%), Borneol (9.5%), Germacrene-D (2.5%), β-Pinene (1.7%), β-Myrcene (1.6%), Caryophyllene (1.3%) and Linalool (1%) were also present in the essential oil. The percentage of 1,8-cineol was found to be 6.36%, 1,8-Cineol 4.7% and 14.5% in essential oil of *M. spicata*[173], [116], [106]. Caryophyllene has been reported in India to be 2.35% in the essential oil [94]. α-Terpineol has been reported in its essential oil as well (Rios, 2010).

The variation in chemical constituents of essential oils might be due to the variation in the *mentha* species when cultivated. The composition of *mentha* essential oil directly affects the effectiveness of biological activities which have displaces difference in its constituents depending on the growing area whereas chemistry of *mentha* oil is complex and variable as mentioned above. The seasonal changes may account in the variation in chemical constituents of plants grown at different parts of the world .Chemical constituents with antioxidant activity present in plants determine the role of plants in prevention of many degenerative diseases. The human food suppliments including herbs, contain higher amount of compounds that are capable of deactivating free radicals . The plant extracts were assessed for their antioxidant potential by three methods that includes DPPH scavenging, ABTS scavenging and H_2O_2 scavenging bioassays. The methanolic extracts of the plants showed significant scavenging of free radicals. By comparing DPPH and ABTS bioassays results. It was assumed that the antioxidant potential of the three plant extracts is much increased in case of DPPH bioassay as compared to ABTS bioassay. Among the three plants, the antioxidant potentialof *M. longifolia* was very significant followed by *M. arvensis* and the extract of *M. spicata* be the least in antioxidant potential as compared to other tested extracts (Rios, 2010).

Similarly in the current research work, *M. arvensis* has shown a considerable amount of antioxidant potential. However the studies conducted in Malaysia and Bangladesh reavealed the good antioxidant potential and anti-inflammatory activity of *M. arvensis*. *M. spicata* also possess the antioxidant potential but less as compared to other two plant extracts (Packer *et al*., 2004).

Scavenging of H_2O_2 was exhibited by the plant extracts was dose dependent. The total radical scavenging activity (superoxide and hydroxyl radical) of whole plant extract of *M. spicata* was found to be quite significant and showed effectively in dose dependent manner and suppressed the production of H_2O_2 (37%) at the dose concentrartion 300 µg/mL which is a characteristic of chain-breaking antioxidants, and has been observed in oxidation of linoleic acid emulsion with extract (Upadhyay, 2015).

Flavonoids, phenolic acids and phenolic diterpenes are the examples of phenolic components with antioxidant properties. Scavenging of extracts may be attributed to their phenolic constituents which can donate electron to H_2O_2, thus neutralizing to water. Although H_2O_2 is not very reactive, it can sometime cause cytotoxicity by rising hydroxyl radicals in the cells. Removal of H_2O_2 by food systems is very important (Valko *et al.*, 2006).

Antimicrobial activity of essential oil of *M. arvensis, M. longifolia* and *M. spicata* in hexane has been tested against Gram +ve and Gram –ve bacterial strains. The selected herbs have been used traditionally as a remedy for respiratory diseases like bronchitis, sinusitis, tuberculosis and common cold. These activities are mostly due to presence of oils like menthol, carvone, limonene etc. The results indicate that essential oils exhibit significant antibacterial activity. However, essential oil of *M. arvensis* showed much activity against *S. aureus* and *B. cereus* at concentration 100µg/mL and 200µg/mL as compared to other tested bacterial strains while its antibacterial activity against *S.typhi* at 200 µg/mL was also higher (Upadhyay, 2015).

The antibacterial study indicates that the plant essential oils have tremendous antibacterial activity at higher concentrations against various bacterial strains that are the major causative agents mainly of stomach problems. The finding of zone of inhibition was found to be higher than the results reported by other authors .

Cytotoxicity refers to the ability of certain chemicals to destroy the living cells in the body. By a cytotoxic compound, healthy living cells either induce necrosis (accidental cell death) or apoptosis (programmed cell death). Brine shrimp lethality bioassay used for *M. arvensis, M. longifolia* and *M. spicata* indicates that all the plant extracts are very less toxic and are suitable to be used in folk medicine (Skehan *et al*., 1990) which also indicates importance of *mentha* extracts for its application in pharmaceutical industry for development of drugs.

CONCLUSION

The selected mentha species constitutes a considerable amount of phytochemicals including flavonoids, phenolics, annins and saponins. The major compounds of M. arvensis essential oil were carvone (84.3%), 1,8-cineol (5.3%) and limonene (3.2%) while major compounds of M. longifolia were piperitone oxide (54.2%), menthone (9.7%), 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (8.4%), borneol (4.6%) and menthol (3.3%). 2-Hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (47.1%), 1,8-cineol (12%), *α-pinene* (9.7%), borneol (9.5%) and germacrene-D (2.5%). The organic compounds obtained by GC-MS are likely to be the main antioxidants.

The antibacterial study indicates that plant essential oils showed better activities at higher concentration against tested strains. The zone of inhibition were found to be much relatable to the reported data. Brine shrimp lethality bioassay indicates that these plants are non-toxic and are suitable for use as folk medicine and may be used in the preparation of drugs. Essential oils of M. arvensis and M. longifolia are a potent source of mosquito repellents.

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WHAT IS VACCINE?. HOW DOSE VACCINATION SAFE HUMAN POPULATION BY CONTROLLING VIRAL DISEASES *A Review article*.

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ABSTRACT

A vaccination is a biological preparation that gives active acquired immunity against a specific infectious disease. Vaccines are formed from kill form of microbes, its toxins and surface protein of microorganisms which causes disease. There are four types of vaccines (a) live attenuated vaccines (b) inactive vaccines (c) subunit recombinant polysaccharide and conjugated vaccines as well as (d) Toxoid vaccines. Therefore vaccines can be in the form of adjuvant, valence, excipients and preservatives. There are many types of viral vaccines for different disease such as measles, mumps, rubella, viccinia, varicella, zoster etc. The current example of viral disease is COVID 19 epidemic, which is causing serious health conditions in human population throughout the world. There have been 222,788,994 confirmed cases of COVID, with 4,600327 deathsand recorded cases were 199,314,577 as reported by WHO on September 8, 2021. To deal with this problem, experts of viral diseases from all over the world, particularly in wealthy countries, are frantically trying to create vaccines that could have the ability to treat coronavirus sufferers. However, a large portion of the world's population continues to wait for their fantasy medications to arrive in the markets.

Keywords; Vaccine, Preparation, Patient and COVID-19 virus

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INTRODUCTION

The health of a country's population has an impact on its economic progress. Capital, health, and education are among the most important variables in a country's development. Investments in the domains of health and education would hasten economic development (Ahmad,2021). Individuals' contributions to production and growth will increase if they are healthy. Our way of life has come to an almost complete halt as a result of the global coronavirus epidemic, which has already inflicted immeasurable devastation and sorrow. In every corner of the globe; ; the pandemic's economic and sociological consequences will be severe and long-lasting. The pandemic has revealed that progress made in addressing poverty, hunger, good health, and well-being may be compromised unless the international community addresses global environmental threats that have the same potential to seriously undermine the systems that allow humanity and the planet to survive and thrive(Bakarey, 2021). Because of the COVID-19 pandemic, many of us are remaining at home and engaging in less social engagements. (Fedson, 2005)

Developmental economics

Vaccine development and production are both costly and susceptible to commercial failure. Many vaccine-preventable diseases, such as HIV, malaria, and tuberculosis, are primarily found in underdeveloped countries. Pharmaceutical and biotechnology firms have little incentive to develop vaccines for these diseases since the revenue potential is so small (Fig 1). Even in more wealthy nations, financial benefits are typically limited, and financial and other risks are considerable. Most vaccine development has thus far relied on government, university, and non-profit "push" funding. Many vaccines have been demonstrated to be both cost-effective and beneficial to public health in general. A number of smaller groups work on vaccine research and development(Ahmad, 2021).

An oligopoly of major producers provides vaccines on a vast scale. No health expert would publicly criticize drug corporations at this critical point with coronavirus, but many privately grumble that pharm is a significant roadblock in creating lifesaving vaccinations.Supply shortages and high healthcare costs connected with paying employees to hunt for difficult-to-find medications have also emerged from the concentration and monopolisation of medication manufacture.(Bilal and Iqbal, 2020)By 2010, five multinationals, GlaxoSmithKline,

SanofiPasteur, Pfizer, Merck, and Novartis, controlled 80 percent of the worldwide vaccine market: GlaxoSmithKline, SanofiPasteur, Pfizer, Merck, and Novartis. Vaccine development is not a priority for Novartis. The oligopoly's longevity is aided by patents on critical production processes.(Weniger *et al*., 1999)

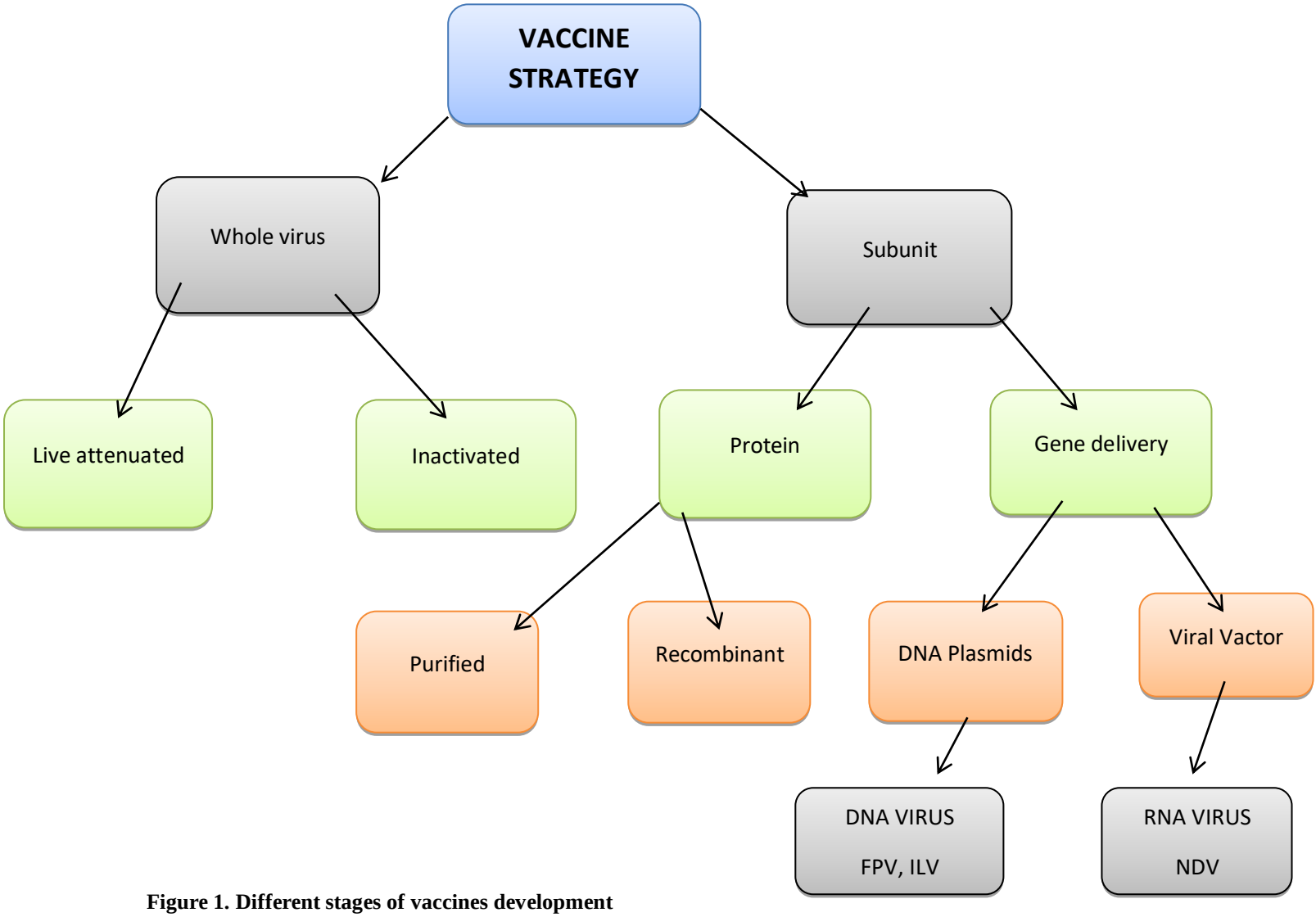

Figure 1. Different stages of vaccines development

Market incentives

Pharmaceutical firms have no financial incentive to test vaccines that benefit primarily the poor. Vaccines made for affluent countries may have short expiration dates and require refrigeration until they are delivered and given in several doses, which may be difficult to do in remote areas. In other cases, the vaccination has never been tested to check if it still works if the criteria aren't satisfied (for example, if it keeps its potency for several days without being refrigerated). Pharmaceuticals, including vaccines, are almost always developed using public funding, but profits, price, and availability control are legally handed to pharmaceutical companies. (Diane*et al.*,2017).Large pharmaceutical firms spend the majority of their income on dividends and stock buybacks, which increase CEO compensation, as well as lobbying and advertising. Investing in the Rand D at a low rate is regularly marketed as an inducement to investors. Rather of being created in-house, innovation is usually purchased along with the small businesses that created it.(Hilleman, 2020) The pharmaceutical industry's financialization focus, particularly in the United States, has been highlighted as a barrier to innovation. With accepting donations of generally unaffordable vaccine, there have been ethical concerns highlighted.(Oyarzún and Kobe, 2016)

NEW VACCINE CONCEPTS

Particles that look like viruses

VLPs (viral like particles) are recurring structures with a high density of viral capsid proteins that are well structured. Because of the high concentration of capsid proteins, there are many conformational viral epitopes, which can elicit strong immune responses. VLPs are formed by the self-assembly of viral capsid proteins in the absence of any infectious nucleic acids from the virus. They may be a safer alternative to the attenuated viruses commonly used for immunisation due to their full inability to replicate.(Dongarwar and Hamisu, 2021).VLPs have been shown to elicit strong immune responses even in the absence of an adjuvant. The virion's structural proteins are frequently arranged in a compact, well-ordered form that is considered to be recognised as a PAMP. As a result, one way to enhance the immunogenicity of viral antigens is to distribute them in multimeric form and as virus-like particles (VLPs). VLPs produced from enveloped and non-enveloped viruses can be used to immunise against the same virus or can be modified to incorporate epitopes from another pathogen. VLPs are regarded highly safe because

they do not contain any genetic material and have a higher immunogenicity.Recombinant viruses have been used as vectors for protein production and vaccination for decades. The list of viral families being studied as vaccination vectors is simply too extensive to list here, and the topic has recently been explored elsewhere. Viruses can be changed to enhance their safety and immunogenicity by eliminating virulence factors, switching envelope proteins to change tropism, and removing non-essential genes to increase coding capacity. The antigen is formed in the setting of a genuine viral infection, which induces innate immune responses that are required for the full development of adaptive humoral and T cell immunity.(Koirala*et al.*, 2020).Potential disadvantages include competition with the vector's immune-dominant antigens or loss of efficacy in the face of pre-existing immunity to the vector. Although it is not required for cell culture replication, the non-structural protein NSs is a crucial virulence factor that governs the host's immune response. By applying reverse genetics to attenuated strains and proving safety and immunogenicity in mice and lambs, several organisations have created viruses that lack NSs.(Oyarzún and Kobe, 2016)

Recombinant proteins and synthetic peptides

Using recombinant methods or chemical synthesis to deliver a viral antigen is a safe strategy to elicit immune responses.Recombinant protein vaccines can have other benefits in addition to safety: First, manufacturing does not need pathogen manipulation, which eliminates the possibility of inadvertent escape as well as the challenges of bio-safety and bio-containment. Second, even with minimal information about the disease, vaccine candidates can be developed. Third, subunit vaccines can be used to bypass the immune system's natural predilection for highly variable epitopes and direct immune responses to conserved, broadly protective epitopes. Fourth, because particular antigens elicit responses distinct from those evoked by natural infection, these vaccination methods might be used as DIVA (Differentiating Infected from Vaccinated Animals) vaccines with a serological test to distinguish infected from vaccinated animals. (Leroux-Roels*et al.*, 2011)The fundamental problem of subunit vaccines is that they are frequently poor immunogens because they do not detect Pathogen-Associated Molecular Patterns (PAMPs) and so do not trigger innate immune responses, which are required for the full development of acquired immunity. They must be administered in an immunogenic form and/or

be accompanied with a potent agonist to increase immune responses to conserved epitopes.(Wallis *et al.*, 2019)

Nucleic acid vaccines

DNA vaccines provide a number of advantages for vaccines against novel viruses: plasmids expressing a viral antigen may be produced fast, even if only a portion of the pathogen's sequence is known. When antigen is generated in vivo, it triggers both humoral and cell-mediated immune responses. DNA vaccines are more stable than other types of vaccines and can be produced in big quantities in a short amount of time at a cheaper cost, both of which are crucial characteristics for a vaccine that must be used in remote locations. DNA vaccines are also considered to be highly safe, perfect for DIVA applications, and immune to anti-vector immunity. (Maiyegun*et al.*, 2021)The major barrier to the development of DNA vaccines is their low immunogenicity. DNA vaccines are frequently used in combination with other immunisation platforms in prime-boost methods. Replicon vaccines are made up of defective RNA genomes that are capable of replicating and expressing encoded proteins but not of forming infectious virus particles. Antigen-specific humoral and cellular immune responses can be induced by using these plasmids to encode a viral antigen. These discoveries spurred a flurry of research into DNA-based vaccines for a number of illnesses, including influenza, HIV, and the lymphocytic choriomeningitis virus (LCM).RNA-based vaccines have gained popularity in recent years due to the limitations of DNA vectors. They are inexpensive and can be mass-produced fast, much like DNA-based vaccines.(Oyarzun and Kobe, 2016; Leitner,2020)

However, RNA's fragility and poor in vivo dispersion have historically restricted its application. Several structural modification methods have been utilised to increase the intracellular stability of RNA molecules. Because RNA, unlike DNA, does not require targeting to and entrance into the nucleus, cell entry is the most significant barrier that RNA vaccines must overcome. This can be addressed by including polycationic carrier molecules into the formulation, which can condense and protect the RNA while also allowing its fast absorption by cells.(May,2005).

Conjugate vaccines

Antigens, both polysaccharide and protein-based, are found in vaccines containing live, attenuated, or inactivated pathogens. It's possible, though, that just a few number of them are

required to elicit protective immunity. This logic has been extended to proteins by the realisation that each protein contains hundreds of possible immunogenic epitopes, not all of which are necessary. (May, 2005)As a result, peptide-based vaccinations have aroused attention. Antigenic epitopes on a protein, on the other hand, are more than just a string of amino acids since the peptides used must mimic the structure of the immunogenic epitope in the native protein. For identifying and mapping the conformation of immunogenic epitopes inside proteins, computational modelling has shown to be a valuable technique. Because peptide or polysaccharide-based vaccines are less immunogenic than those present on the surface of a pathogen, they must be given with an adjuvant. (Sing *et al.*,2021)Another method is to conjugate the antigen to a second 'helper' protein or polysaccharide that has been proven to increase immunogenicity; however, the immune response may be misdirected to the helper molecule. To get around this problem, some people use carrier systems like liposomes to separate the target and helper parts of the vaccination, or they use precise matching and orientation of the target and helper sections of the vaccine.(Metz*et al*., 2009).

Cellular vaccines

Due to the history of success of vaccination using live attenuated viruses, inactivated viruses, or bacteria, attempts to adopt a similar methodology to vaccine against cancer were undertaken. Attenuated tumour cells have been provided to induce an immune response against particular kinds of tumors. Whole cell vaccines have been utilised in two ways: autologous and allogeneic. Autologous cell vaccines have been investigated in cancers such as lung, colorectal, melanoma, kidney, and prostate cancer. Autologous cell vaccines, on the other hand, are limited to a few types and stages of cancer due to the need to prepare a large portion of the patient's tumours.(Sorochi*et al.*, 2021).To increase immune activation, several whole cell vaccines have been genetically designed to stimulate the production of cytokines, chemokines, and co-stimulatory molecules. The patient's own immune cells, especially dendritic cells, are used in another kind of cellular vaccination. Tumor-associated antigens or nuclei are loaded into a patient's autologous dendritic cells while they are being treated with immunoadjuvants to create dendritic cell vaccines. Dendritic cell vaccines have been tried in clinical trials against prostate, melanoma, kidney, and glioma cancers.This vaccination regimen involves the collection of the patient's peripheral blood mononuclear cells, as well as cell culture processing and reinfusion,

which are both time-consuming and expensive operations. While these cell-based methods are fascinating, they do not appear to contribute to the shift away from live and attenuated vaccines toward vaccines with reduced complexity and manufacturing costs that are better suited to treating large populations while lowering health-care costs.(Oyarzún and Kobe, 2016)

Recombinant bacteria as vaccine vectors

In addition to being commonly utilised to make recombinant subunit vaccines, bacteria can be used as vectors for in vivo delivery of antigens or DNA. This platform's potential benefits include cheap cost and simplicity of scaling-up manufacturing, the availability of well-characterized attenuated strains, the vector's stimulation of innate immunity, and effective transport to antigen-presenting cells.Listeria, Salmonella, Lactococcus, and Bordetella are among the genera being investigated as vaccine vectors. Recombinant bacteria can be utilized as live vaccines, inactivated germs, or even bacterial ghosts with no cytoplasm. In mice, recombinant Lactococcuslacti expressing the SARS-coronavirus N protein has been demonstrated to produce antibodies.(Wallis *et al.*, 2019)

Vaccines against Bioterrorism

The potential use of biological organisms as weapons of war or vehicles for terrorism has piqued the curiosity of both the lay and scientific press in recent months. Movies like Outbreak, famous books like The Cobra Event and The Eleventh Plague, and numerous press accounts of groups like the Aum Shinrikyo cult in Japan have raised public awareness of the threat posed by biological agents used for nefarious purposes. Military and civilian law enforcement organizations have begun training crisis-response teams in order to prepare for biological disasters. The possibility of a biological attack is now frequently considered in "war-gaming" exercises and counter-terrorism planning by agencies such as the Department of Defense (DoD), the Centers for Disease Control and Prevention (CDC), the Federal Emergency Management Agency (FEMA), the Federal Bureau of Investigation (FBI), and others. Despite our best efforts and diplomatic precautions, the risk of biological agents being utilized in warfare or terrorism looks to be very significant. The relatively low level of technological skill and cost necessary to manufacture a biological weapon compared to other weapons of mass destruction are among the reasons for this such as hemical and nuclear arms. With this in mind, it appears unlikely that enhanced awareness, advanced surveillance and speedy crisis response will completely prevent

all biological aggression attempts(Wirsity*et al.*, 2021). As a result, vaccines will likely continue to be one of the greatest defenses, especially in a military setting, and this will necessitate the development of new and improved vaccines and therapies against the relatively small number of effective biological warfare agents. Although civilian planners are unlikely to use such vaccines in the near future, they may explore vaccination as a consequence management strategy following a biological terrorist attack on civilians in select instances.(Hilleman, 2002)

Biological warfare agents are classified in three ways: (1) operationally, as lethal or incapacitating agents with or without secondary transmission potential; (2) by intended target, as antipersonnel, antianimal, antiplant, or antimateriel; and (3) by type, as replicating pathogens, toxins, or bio modulators.

MATERIAL AND METHODS

Different information accessible for clinically developing vaccines, their effects on the human body, and their market values has been compiled into a single text for readers' instant awareness. A vaccine takes an average of 12-36 months to make before it is ready for distribution. Vaccines are complex biological products that take a long time to manufacture and test. Successful manufacturing of high-quality vaccines necessitates international standardization of starting materials, production and quality control testing, and the establishment of high expectations for regulatory oversight of the entire manufacturing process from beginning to end, all while acknowledging that this field is still in its infancy. All components, manufacturing processes, testing methods, reagents, and standards must adhere to the Good Manufacturing Practices guidelines (GMP). To ensure vaccine identity, purity, sterility, efficacy, and safety, these stringent quality standards include ad hoc pharmaceutical quality systems, quality assurance measures and processes, numerous quality controls at each stage, and a suitable infrastructure and activity separation. Production lead times for complex multivalent vaccinations might exceed 36 months. Two of the six vaccines are now being tested in humans (Yang *et al.*,2016). Five are created by infecting human fetal cells (factories) with adenoviruses that carry genes from SARS-CoV-2, the virus that causes COVID-19. The sixth vaccine, which could enter human trials this summer, is a protein subunit vaccine.(Bilal and Iqbal, 2020).

Different stages of Vaccine development

Vaccines development

Vaccine development is the process of taking a new antigen or immunogenic found during the research phase and turning it into a finished vaccine that can be tested in preclinical and clinical studies to establish the vaccine's safety and efficacy.

The stages of a vaccine's development are as follows:

Exploratory stage

Basic lab research and the identification of natural or synthetic antigens, which alert the body to hazardous microorganisms, often lasts 2-4 years during the exploratory phase (Bollmann). If the vaccine proves to be effective in the exploratory phase, it will be tested on animals.

Human clinical trials are divided into three phases: phase I, phase II, and phase III, with official regulatory approval necessary in some countries for any of these studies.

Pre-clinical stage

A medication candidate may also be examined in lab animals at this stage before going on to Phase I trials. Vaccines like the oral polio vaccine were first tested for adverse effects and immunogenicity in monkeys, non-human primates, and lab mice. Before a vaccine is approved, it must undergo a clinical study to establish its safety and efficacy. High throughput screening and identification of the right antigen to trigger an immunological response may be included in preclinical investigations of vaccine candidate medicines.

Clinical development

Clinical development is divided into three stages. Small groups of people are given the experimental vaccine during Phase I. The clinical research is expanded in Phase II, and the vaccine is given to persons who have characteristics (such as age and physical health) that are similar to those who will benefit from the new vaccine (Fig 2). Thousands of people are given the vaccine in Phase III, and it is examined for efficacy and safety. After a vaccine is approved and licensed, it is subjected to Phase IV formal, ongoing trials.In order to evaluate the vaccine candidate's safety, the Phase I research entails introducing it to healthy persons. In order to

evaluate the vaccine candidate's safety, the Phase I research entails introducing it to healthy persons. Healthy volunteers are enrolled in a Phase I immunization trial and are given either the candidate vaccine, a "control" therapy (such as a placebo or an adjuvant-containing cocktail), or a proven vaccine (which might be intended to protect against a different pathogen). The major goal of the test is to look for signs of safety (no adverse events) and evidence of an immunological response.(Leroux-Roels *et al*., 2011)

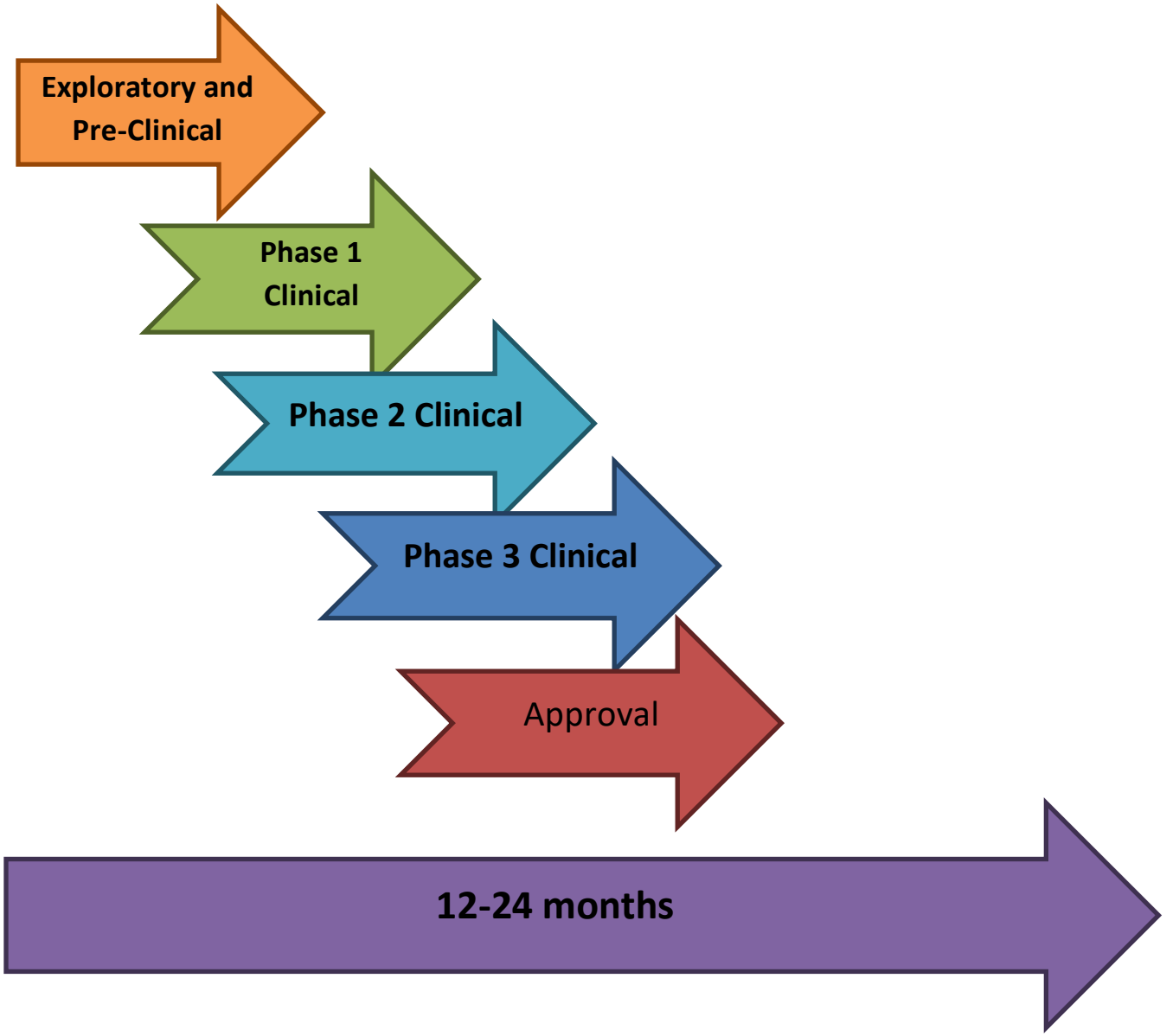

Figure 2. Clincal trial of vaccines

Regulatory review and approval

Nearly every stage of vaccine development, manufacture, and marketing clearance involves regulatory difficulties. Regulations apply from the time a vaccine is designed and clinically tested, through manufacture, and distribution to the general public (May, 2005).

Manufacturing

Vaccine production is a lengthy process. Vaccines take anything from 7 to 36 months to manufacture, package, and deliver to those in need. It entails testing each batch of vaccination at each stage of its trip, as well as repeated quality monitoring of batches by various authorities throughout the world (Lerous-Roels*et al*.,2011)

Quality control

Vaccine quality control used to rely on a range of testing procedures to guarantee that the products were safe and effective. These techniques were created for vaccines whose safety and efficacy were determined after years of research. However, as vaccine manufacturing technology has advanced. Tests can now detect potential risks with a sensitivity that wasn't conceivable just a few years ago, and a growing number of physicochemical approaches allows for considerably improved product characterization. Vaccine regulation includes a number of different measures in addition to sophisticated tests to verify safety. These include supplier audits for characterization of starting materials, cell banking, seed lot systems, adherence to GMP principles, independent release of vaccines on a lot-by-lot basis by national regulatory authorities, and enhanced pre- and post-marketing surveillance for possible adverse events after immunization.(Metz *et al.*, 2009)

RESULTS AND DISCUSSION

Successful vaccine manufacturing necessitates international standardization of starting materials, production and quality control testing, and the establishment of high expectations for regulatory oversight of the entire manufacturing process from start to finish, all while acknowledging that this field is constantly changing. 1. All components, production processes, testing methods, reagents, and standards must adhere to the Good Manufacturing Practices (GMP). Pharmaceutical quality systems, quality assurance techniques and processes, multiple quality

controls at each level give guarantee vaccine identity, purity, sterility, efficacy and safety, and suitable infrastructure are all part of these stringent quality criteria (Sorochi*et al.*,2021)

- The vaccine's efficacy or performance is determined by a number of factors, including the disease itself (for some diseases vaccination performs better than for others)
- The vaccination strain (some vaccines are specific to, or at least most effective against, particular strains of the disease)
- Whether the immunization schedule was followed correctly.
- A person's unique reaction to vaccination; some people are "non-responders" to specific vaccines, meaning they do not produce antibodies even after being properly vaccinated.
- Ethnicity, age, or genetic susceptibility, to name a few.

The following are important factors to consider when determining the efficacy of a vaccination programmer:

1. Carefulmodeling to predict the impact of an immunization campaign on disease epidemiology in the medium to long term
2. Ongoing surveillance for the relevant disease following the introduction of a new vaccine
3. Maintaining high immunization rates, even when a disease has become rare.

Vaccines for more than 20 life-threatening diseases are now available, allowing individuals of all ages to enjoy longer, healthier lives. Every year, vaccines prevent 2-4 million deaths from diseases such as diphtheria, tetanus, pertussis, influenza, and measles. Immunization is an indisputable human right and an important component of primary health care. It's also one of the most cost-effective health investments available. Vaccines are also important for preventing and controlling infectious disease outbreaks. They are essential in the fight against antimicrobial resistance and support global health security(Wirsiy*et al.*,2021). Despite significant advances, far too many people around the world – including approximately 20 million infants each year – lack adequate immunization access. Progress has slowed or even reversed in some nations, and there is a serious danger that complacency will destroy previous successes (Wallis*et al.*,2019)

Vaccines contain pure materials obtained from dead or inactivated organisms. Vaccines come in a variety of shapes and sizes. These are many approaches of reducing the risk of sickness while maintaining the ability to elicit a positive immunological response.Monovalent (also known as

univalent) and multivalent (also known as multivalent) vaccines are available (also called polyvalent). A monovalent vaccine is intended to protect against a single antigen or microbe. A multivalent or polyvalent vaccine protects against two or more strains of the same microbe, or two or more germs altogether.A multivalent vaccine's valiancy might be indicated by a Greek or Latin prefix (e.g., tetravalent or quadrivalent). In rare cases, a monovalent vaccination may be useful for inducing a strong immune response fast. When two or more vaccinations are mixed in the same formulation, it's possible that they'll create problems.(Sing*et al.*,2021)This is especially frequent with live attenuated vaccines, when one vaccine component is more strong than the others, limiting the growth and immune response to the others. The number of serotype 2 viruses in the trivalent Sabin polio vaccine has to be reduced to avoid interfering with the "take" of the serotype 1 and 3 viruses. Current dengue vaccines, in which the DEN-3 serotype predominates and inhibits the response to the DEN-1, 2, and 4 serotypes, have been discovered to have this issue.People who have had a severe reaction to adsorbed tetanus toxoid may be given the basic vaccination instead when it's time for a booster.(Yang *et al.*, 2016)

Preservatives may be added to vaccines to avoid contamination by bacteria or fungi.
Preservatives may be utilized at many phases of vaccine manufacture, and the most advanced measurement methods may identify residues of them in the completed product, just as they may in the environment.

In addition to the active vaccine, the following excipients and residual production chemicals are included or may be present in vaccine formulations:

- Adjuvants are employed, such as aluminum salts or gels.
- Adjuvants are included to vaccines to stimulate a faster, more powerful, and longer-lasting immune response, allowing for a lower vaccination dose.
- Antibiotics are used in certain vaccinations to prevent bacteria from growing during manufacturing and storage.
- Because influenza and yellow fever vaccinations are made from chicken eggs, they include egg protein. Other proteins might be present as well.
- Toxoid vaccinations utilize formaldehyde to inactivate bacterial products.
- Stabilizers like monosodium glutamate (MSG) and 2-phenoxyethanol are used in a few vaccines to keep them stable whether they're exposed to heat, light, acidity, or humidity.

- Thiomersal is a mercury-based antibiotic that is added to multidoseimmunisation vials to prevent contamination and the growth of potentially harmful pathogens.

Epidemic response

In the past, pharmaceutical firms' commercial dominance has caused epidemic solutions to be delayed. As a prerequisite of manufacturing vaccines, manufacturers have successfully negotiated favorable arrangements with governments, including market assurances and indemnity(Maiyegun*et al*., 2021). This has resulted in months of delay in certain epidemic responses and the complete absence of reactions in other pandemics. Some intellectual property problems, such as in the instance of rVSV-ZEBOV, also impede vaccine research for epidemic preparation.

Demand

Vaccines make up just 2% to 3% of the worldwide pharmaceutical business, but they are growing at a pace of 10-15% each year, far faster than other medications (as of 2010). Vaccine demand is increasing in emerging nations, due in part to international vaccine funders (UNICEF purchased half of all vaccine doses worldwide in 2012). Vaccines are becoming the financial engine of the pharmaceutical industry, and new business models may develop as a result. Vaccines are being sold in the same manner as pharmaceuticals.Vaccines give public-private partnerships, governments, and philanthropic donors and foundations new financial opportunities (such as GAVI and CEPI). Thanks to philanthropic funding, vaccines are now being pushed out to large emerging markets less than 10 or 20 years after they are developed, and for the duration of the patent owner's patent validity term.**(**Hilleman, 2020; Koirala et al.,2021**).** Vaccinations that are newer are much more costly than previous vaccines. Vaccines are becoming more profitable in lower-income nations. The financial component of vaccine development is one of the most difficult aspects: Many vaccine-preventable diseases, such as HIV, malaria, and tuberculosis, are primarily found in underdeveloped countries. Pharmaceutical and biotechnology firms have little incentive to develop vaccines for these diseases since the revenue potential is so small.(Dongawar and Hamisu,2021).

Veterinary Vaccine

Animal vaccination is used to prevent illness in animals as well as disease transmission to humans. Both dogs and livestock are vaccinated on a regular basis. In certain situations, wild populations may be vaccinated. It has been used to decrease rabies in raccoons by spreading vaccine-laced food in disease-prone areas. In regions where rabies is present, rabies vaccination of dogs may be required by law. Vaccinations for dogs include canine distemper, canine parvovirus, infectious canine hepatitis, adenovirus-2, leptospirosis, bordatella, canine para influenza virus, and Lyme disease.Veterinary vaccines have been given to people, whether on purpose or by mistake, resulting in disease outbreaks, most notably brucellosis. However, such incidents are seldom recorded, and little study on the safety and effectiveness of such therapies has been conducted. Since the advent of aerosol vaccination in veterinary clinics for companion animals in recent years, human exposure to diseases that are not normally carried in humans, such as Bordetellabronchiseptica, has definitely increased. In certain cases, like as rabies, animal immunisation against a disease can be hundreds of times less expensive than human vaccination. (Bakarey,2021).

DIVA vaccines

Infected and vaccinated animals can be differentiated using DIVA (Differentiation of Infected from Vaccinated Animals) vaccines, also known as SIVA (Segregation of Infected from Vaccinated Animals). In the field, microorganisms contain at least one epitope fewer than DIVA vaccines. With the use of a diagnostic test that identifies antibodies against that epitope, we can make that distinction. J.T. van Oirschot and colleagues at the Lelystad-based Central Veterinary Institute, produced the first DIVA vaccinations (previously known as marker vaccines, and since 1999 known as DIVA vaccines) and companion diagnostic tests.They detected deletions in the viral genomes of some current pseudorabies (also known as Aujeszky's sickness) vaccines (among which was the gE gene). In the same vein, DIVA vaccines and diagnostic assays for bovine herpesvirus 1 infections have been created(Koirala*et al*., 2020). In a number of countries, the DIVA technique has successfully eradicated the pseudorabies virus. Swine populations were aggressively vaccinated and monitored using a companion diagnostic test, with unhealthy pigs being killed. The virus that infects cattle is known as bovine herpesvirus 1. Furthermore, DIVA vaccines are commonly used in clinical practise. The DIVA principle has been used to a number of infectious diseases, including classical swine fever, avian influenza,

Actinobacilluspleuropneumonia, and Salmonella infections in pigs, among others. (Sing *et al.*,2021; Wenige*et al.*,1999; Wirsiy*et al.*,2021).

CONCLUSION

Vaccines are an important part of the protective measures for uniformed military members. Licensed anthrax, smallpox, and plague immunizations are available. The Department of Defense recently initiated an anthrax immunisation campaign across the military, and other anti-biological warfare vaccines may be deployed in the future to protect soldiers, sailors, airmen, and marines. Finally, vaccines against other biological agents, as well as improved vaccines against the agents mentioned above, are at various stages of research. Because the nature of the threat is less explicitly defined, using these immunizations in a civilian setting is more problematic.Vaccines for anthrax and smallpox, for example, may be effective in the post-exposure prophylaxis and management of exposed civilian populations. It can be difficult to develop vaccines for developing infectious diseases. Several recently established vaccination approaches can exactly address these difficulties. Subunit vaccinations, which only contain a fraction of the pathogen's antigens, can produce different protective responses than afflicted animals.Because they contain no infectious disease, there are no severe bio-safety measures, no risk of unintended escape during production, no residual pathogenicity, and no reversion. Using well-defined vaccination platforms with a lengthy track record of safety and efficacy against similar diseases can assist speed up vaccine development for novel and potentially emerging viruses. Each vaccination platform offers advantages and disadvantages that are mostly related to the balance of safety and immunogenicity, as well as the ability to be used multiple times. In order to improve vaccination approaches' safety and immunological qualities

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Volume 1, Issue 2. August, 2021. www.jbbt.org

GUT MICROBIOMES AND ITS EFFECT ON COGNITIVE HEALTH

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ABSTRACT

The human gut microbiomes play important role in brain physiology and pathophysiology. The microbiome of gut linked to the brain through vagus nerve and help in the cognitive development and health. In adults gut microbiomes may affect the nervous system by causing stress, anxiety, depression and cognition increasing clinical and proclinical reports show that gut microbiomes may cause the neurological disorders such as Alzheimer's disease, Parkinson's disease, multiple sclerosis and stroke. Further study help to show us an evidence that how per and probiotic balance the brain functions in healthy and diseased individual. The parental diet also contributes in causing changes in infant microbiome that is high or low fat ratios can effect adversely on neonatal cognitive health later in life. The need of taking a constant supply of nutrients of a fetus from the mother changes with the gut microbiome of mother. Western diet plays a very major

role in gut microbiome in changing the behavioral characteristics for cognitive health. The type of nutrition is a major factor that is related to cognitive impairments or causing obesity and its effects on human physical and mental health. The following review article sheds light on interrelation of gut micro biomes with the host's immunity, while explaining the correlation of intestinal permeability and obesity. Furthermore, the production of enzymes induced by micro biota and their effects associated with systemic inflammation. Secondly, cognitive dysfunctions and importance of leptin concentrations and white adipose tissues in that regard is also explained. Thirdly, the blood brain barrier – BBB and its communicatory network with gut micro biomes is discussed. Finally, a psychiatric perspective of microbiota and brain relation is given while further focusing on the same communication network.

Key words; Gut microbiomes, vagus nerve, Gut brain axis, metabolic signaling, laptine resistance

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INTRODUCTION

The bio directional link between gut microbiome and the nervous system is critical to brain function. The gut is one of the largest organs in the body, performing numerous physiological processes such as nutrition metabolism, absorption, and maintaining the physical barrier of epithelial tissue in the gut (Breit *et al*., 2018; Collins *et al*., 2012). The microbiota-gut-brain-axis change the behavior of person and dysregulation of this axis cause neurological disorders such as Parkinson's disease, Alzheimer's disease, strokes, multiple sclerosis and brain injury (Cryan *et al*., 2020).

Gut microbiome play a key role in the life of neonates. As the mother is the main factor for the supply of nutrition or diet in infants and neonates, so the gut microbiome of mother greatly affects the gut microbiome of infant and cause changes accordingly . The changes may lead to cognitive health of infants and neonates that impairs the cognitive behavioral characteristics in human infants that cause impairment in hippocampus structure leads to mental disorders and changes in other brain functions (Davidson *et al*., 2018) . This type of nutrition is usually avoided because obesity generally affects the cognitive health by changes the brain functions and its impacts are mostly adverse and leads to mental disorders (Cryan *et al*., 2020).

As we all know gut microbiota is actually the biggest symbiotic interaction with the host body. It plays keep roles in the balancing of homeostasis in the intestines . Human Body, including all the mucosal environments, the gut and the skin serves as a host to a huge number of microorganisms, which is collectively known as microbiome. All of this research has demonstrated that the gut microbiome is more than a spectator; it actively interacts with and influences various functions within the host, including metabolism, nutritional responses, circadian rhythmicity, and, most critically, the immune system (Ding *et al.*, 2010).

As we all know, obesity is considered the mother of all diseases, and gut microbiota has been shown to be one of the promoters of obesity and related diseases. As the gut microbiota is regarded as the metabolic entity of the body, it regulates nutritional efficiency, energy balance, and homeostasis, as well as body weight. As a result, any alteration in the normal functioning of the gut microbiota, such as an increase in normal intestinal permeability, results in chronic inflammation, which promotes obesity and other metabolic dysfunctions such as fatty liver disease (Formy-Germano *et al.*, 2019).

MATERIAL AND METHODS

Genetic microbiome profiling

In certain experiment DNA extraction and 16s rRNA was taken from the females candidates having age between 40 to 89 as an observation study.

Cognitive measures

We have four different clinical valid measures of cognitive functions such as verbal fluency test, Deary lie Wald reaction time test (DLRT), mini mental state examination (MMSE) and Cambridge. Neuropsychological test automated battery-paired-associated learning test (CANTAB.PAL).

Associate analysis

In liner mixed effects models R lme4 package is used. Microbiomes traits were different on the base of dependent and independent factors. Such as sample collection methods, gender, family and relatedness. It also depends upon the diet and medicine such as antibiotics used by the person.(Jackson *et al.*, 2018)

Elements of Gut microbiome

The GMB contain commercial and pathogenic bacteria, viruses, fungi, and protozoa and also contain their genetic material, secreted proteins and metabolic end products. It helps to develop the functions of body (Gao *et al*., 2020) . The main phyla are Firmicutes, bactericides, actinobacteria and proteobacteria. 90 percent of gut microbiomes are in firmicutes and bacteroidetes. All these microbes which are present in different phyla release different metabolites, harmonies and neurotransmitters with different subscription to variable cognitive function. When compositions of GMB are imbalance it termed as dysbiosis . It is very difficult to eradicate. Dysbiosis causes so many diseases such as irritable bowel syndrome, diabetes, and CNS disorders.(Øyri *et al*., 2015).

Multiplications of cognitive disease due to GMB

The Gut-Brain axis is act like a two face communication system so it can manage the signals between gut and brain such as; neural, endocrine, immunological and metabolic signaling. The gut microbiomes and its metabolites involve the gut brain axis through following pathways and change the cognitive functions (Hantsoo *et al*., 2019). Firstly hypothalamic-pituitary-adrenal (HPA) is affected by the GMB dysbiosis in which it play major role to manage stress response and cognitive function. In human GMB dysbiosis cause depression and anxiety which is associated with HPA dysfunction. When HPA activity is increase it may affect the memory and learning activities of human (Hasan Mohajeri, 2018).

On another way GMB and metabolites interact with blood brain barrier and enhance the permeability and transportation rate by activating the inflammatory response. Inflammatory markers such as interleukin (IL)-6, monocyte chemo attractant protein (MCP)-1 and other cytokines are releasing from endothelial cells and immune cells through GMB metabolites(Jasarevic *et al*., 2004).

Vagal-nerves link the GMB with CNS and autonomic nervous system (ANS). Vagal nerve is stimulated by the product of GMB such as short chain fatty acids (SCFA). CNS impulse move along the ANS pathway and release neurotransmitter choose gastrointestinal epithelia, mucus layers and mucosa by regulating the permeability, motility and secretion (Krabbe *et al*., 2004).

CNS functions are modified by the variety of neurotransmitter and hormones that are released and consumed by the microorganisms of the GMB. Such as human body serotonin is synthesis in gastrointestinal tract, production of serotonin is affected by the gut microbiota and their metabolites which decrease the neurotransmitter and increased the symptoms (Maffeis and Morandi, 2017).

Prefrontal cortex, an area important for complex cognitive tasks such as planning and decision-making can modify by the activity of GMB in myelination, myelin plasticity, and microRNA. In predevelopment stage level of myelination is lower in germ free mice compared to pathogen free mice in the brain development stage. In all these pathways GMB help to enhance the functions of brain including cognition. (Gao *et al*., 2020)

Modulation of mammalian behavior by Gut microbiota

Depression, anxiety and stress

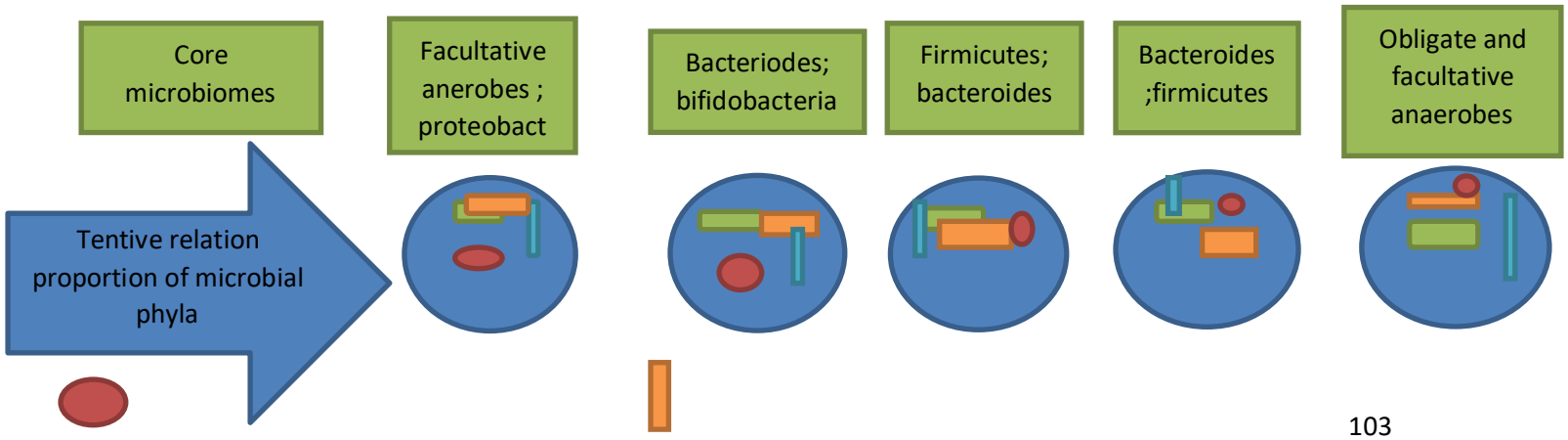
Depression is a worldwide life threatening psychiatric disorder which is caused by the stress. In depression patients level of HPA is disturb so due to this level of corticotrophins releasing factors and cortisol become so high and also proinflammatory cytokines are also found in patient blood plasma. Study proves that gut microbiome directly affect the brain (Mouries *et al*.,2019) . Anxiety and depression is also due to the change of gut microbiome which affect the functions of brain. Sudo et al. study the effect of HPA axis due to microbiome. Their original study based on the stressed germ free mice. They introduce a bacterial strain, Bifidobacterium infantis to remove the side effects of HPA. L rhamnosus has ability to suppress the antidepressants activities in vagotomized mice by reducing the brain function. On other side there are several studies in which gut microbiomes help to reduce depression and anxiety. Probiotic yogurt or multispecies capsule for 6 weeks can reduce the symptoms of depression and anxiety (Myles *et al.*, 2014).

Analysis of fecal sample shows that the microbiome of normal healthy patient is different from the effected. Composition of faeccalibacterium organism may alter from depressed to healthy one. Also number of gram positive and gram negative bacteria in anxiety patient is different. In depressant people number of these bacteria may increase such as; Roseburia, Phascolarctobacterium, Megamonas, Clostridium, Lachnospiraceae incertae sedis, Blautia, Oscillibacter, Parasutterella, Parabacteroides, and Alistipes, whereas are reduced such as; Ruminococcus, Dialister, Prevotella, Faecalibacterium, and Bacteroides (Ochoa-Reparaz *et al*., 2011)..

Cognition

The chance of giving cognitive support to humans may be greater in the age when nutrients are required at high level such as gestation, infancy and older age (Fig 1). .

Study also shows that microbiome may affect the neurodevelopment. Short chain fatty acids are produced by Gut microbiomes and have a main function in brain development and brain activities (Richards *et al*., 2016). Butyrate is important short chain fatty acids which involves in the epigenetic modulation of brain function. Butyrate shows positive effects in Alzheimer's disease through enhancing the memory performance. Butyrate also helps to recover neurodegenerative disorders, including Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, and ataxia (Rromano-Keeler *et al*., 2014).



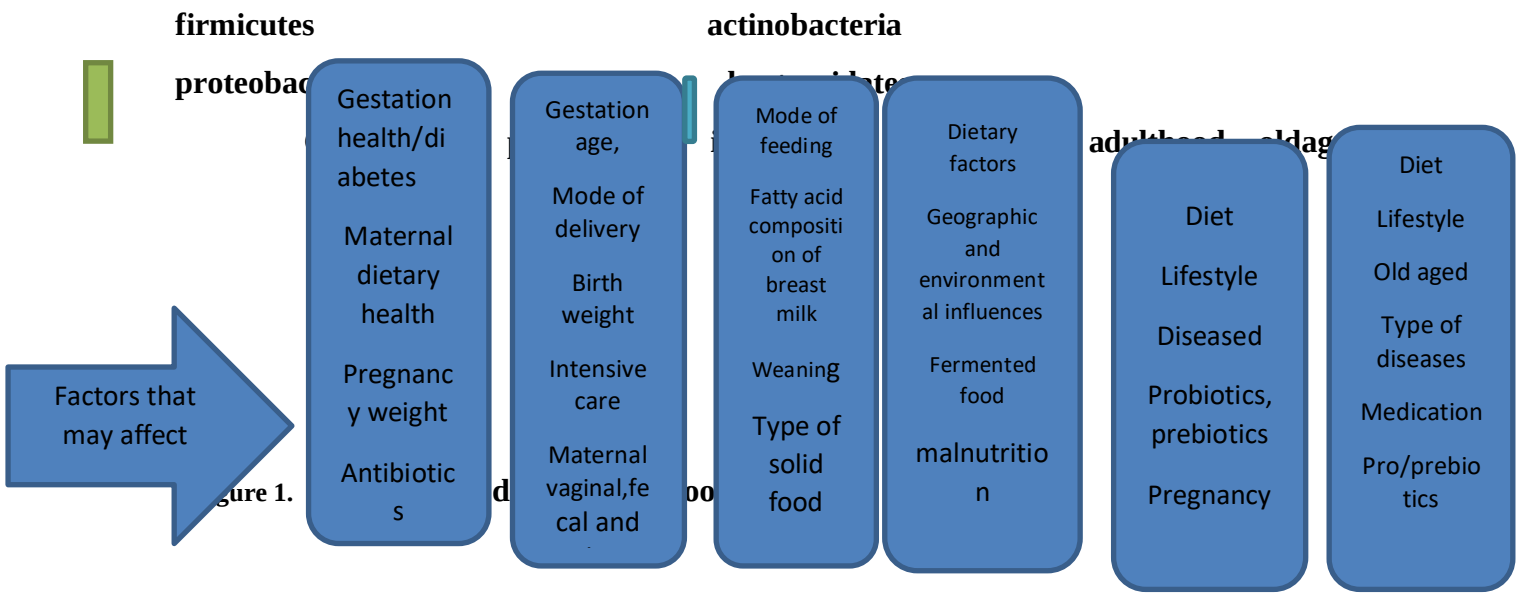


Figure 1: Age-related changes in human gut microbial ecosystem

Mechanistic evidence of microbial influence on neuronal signaling

As we know that gut and brain communicate with each other through vagus nerve.The incoming information from the gut via the vagus nerve to the brain is treated in the nucleus tractus solitaries, which has large projections that include the parabrachial nucleus, which further projects to the prefrontal cortex as well as the amygdala.Study shows that expressions of receptors subunit NMDArec28 are effected by the N-methyl-D-aspartate (NMDA) in amygdala. Tryptophan and serotonin are required during the life span. Tryptophan help in the regulation of serotonin when level of tryptophan is alter due to the consumption of western diet. So in this case the microbiome and neurotransmitter affect the behavior. In mice, study give tremendous result that when mice is treated with antibiotics level of tryptophan in blood and BDNF level in hippocampus is also being disturb (Tappy and Le., 2010) .

Gut microbiomes – Brain interaction in cognitive

Organisms expose to microorganisms early in development to throughout the life. During the development of embryo, it expose to the microorganisms in utero to the birth process where it receive a lot of microbiota which remain retain in GI tract throughout their life cycle. The first microorganisms which are exposed to the human after their caesarian are Bifidobacterium,

Lactobacillus, Enterobacteriaceae, and Staphylococcus while due to vaginal birth dominant flora are Veillonella and Lachnospiraceae.(Hasan- Mohajeri *et al.*, 2018)

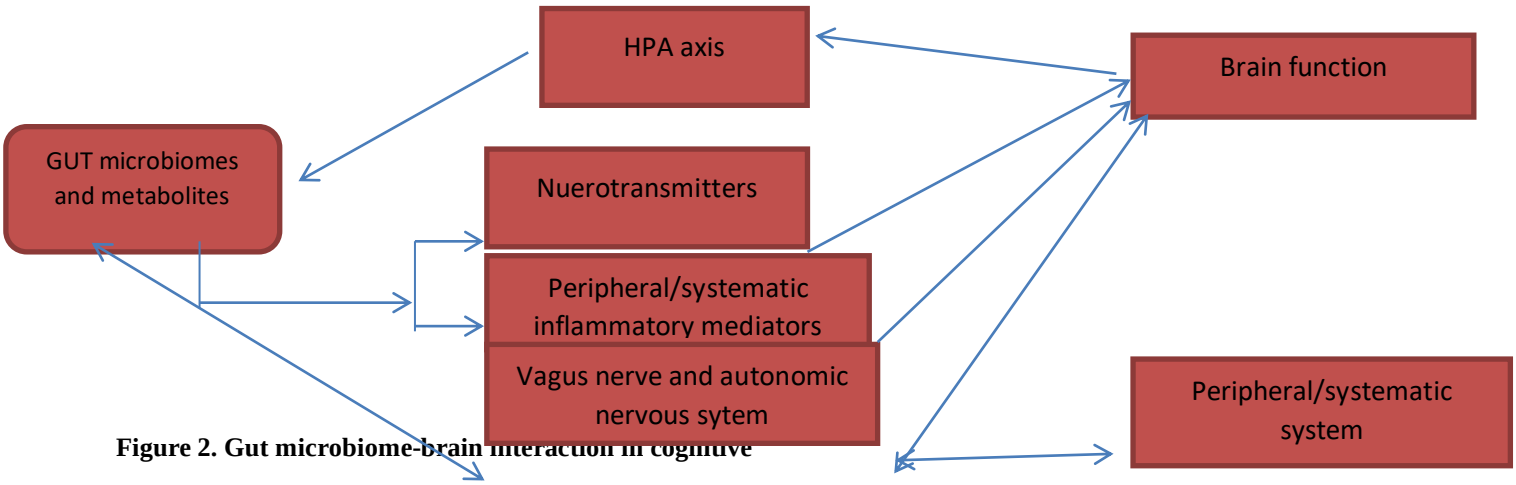


Figure 2. Gut microbiome-brain interaction in cognitive

Gut Brain axis/ second brain

The beneficial bacterial in gut not only provide nutrients to the body but also give them vitamins, energy, and other metabolites. Some good bacteria of GI make a protective biofilms. In recent study it has been proved that bacteria that live in GI may affect the function of brain (Fig 2). The gut brain axis is communication of gut microbiomes with the brain and play an important role in maintaining the brain health or bad microbiota influence the human behavior and may affect the pathophysiology of mental illness (Tito *et al.*, 2012) . System such as central nervous system, the sympathetic and parasympathetic branches of autonomic nervous system, enteric nervous system, immune system and endocrine system ensure the functions of microbiota-gut-brain axis are linked (Tremlett *et al.*, 2012).

In other side irritable bowel syndrome (IBS) and inflammatory bowel disease are two human illnesses which is caused by the faulty gut brain communication. IBS is caused by the gastroenteritis due to the use of antibiotics (Winek *et al.*, 2016). Because of bowl disease it may cause comorbid psychiatric conditions. Germ free mice also show brain abnormalities as compare to normally animals. Level of calcium binding protein calbindin in the enteric neurons in germ free mice or normal mice is same but their expressions in both are different. Immune system is also disturbed with absence of gut microbiomes. As we study germ free mice lack all

the gut bacteria so they show abnormalities in the immune system. Probiotic treatments help to recover the neural functions in B and T cell. (Breit *et al*., 2018)

Microbiota and neurodevelopment

Microbiota-gut-brain axis plays an important role in the mental health. Microbiomes in the gut send signals directly and indirectly to the brain by releasing vitamins, neurotransmitters and neuroactive microbial metabolites such as fatty acids through vagus nerve via nueroimmune and neuroendocrine pathways. Study shows that the animals which are missing the gut microbiota show abnormal brain function because microbiomes active the inflammatory reaction in the brain by regulating the activation of microglial cells and affecting myelination. (Cryan *et al*., 2020)

Role of psychobiome bacteria in gut; probiotics and prebiotic's

Cognitive performance of human and animal model is improved by prebiotics and probiotics. Diet plays a major role for the improvement of GI tract. Consuming a diet which count high, amount of sugar and fats can change the abundance of Bifidobacterium and bacteroides, firmicutes. In recent studies proved that taking acute and chronic prebiotic supplements improved the cognitive performance in humans (Yatsunenko *et al.*,2012).

By taking probiotic supplements which improved the cognitive performance in mouse. But they are not for depression. Probiotic supplements attenuate HPA axis and in hippocampus neurogenesis it decrease the stress and protect the mice. In healthy adults single and mixed probiotic supplements may affect positively.(Cryan *et al*., 2020).

Microbial and neurological disorders

Microbiota-Gut-brain axis plays a major role in the mental health. Some of causes harmful effects on the brain function in human. Because of these illness patients may face many disease as mentioned in the following section

Multiple sclerosis

Microbiota causes so many neurological disorders which are based on immune system. Children's who are affected with the multiple sclerosis, have large scale differences in alpha and beta delivery of gut microbiota as compared to the healthy children without autoimmune disease

up to age 18 years. In this disease, interleukin producing CD4 T cells are affected by the immunomodulatory effects of gut microbiota (Richards *et al.*, 2016).

Parkinson's disease

According to seminal research, Parkinson's disease occurs due to aggregation of alpha synuclein protein in brain and also in mucosal and submucosal nerve fibers and ganglia of Parkinson's patient. Alpha synuclein is move from gut to brain through vagus nerve (Oyri *et al.*, 2015) . Old rat has more amount of amyloid like produced by the bacteria so increase alpha synuclein in their blood as compare to Young.

Alzheimer's disease

Neuroinflammation and neurodegeneration seen in the patients of Alzheimer's disease. According to the studies, Escherichia coli and shigella bacterial taxa is increase in the fecal sample of Alzheimer's disease patient (Jakson *et al.*, 2018).

Stroke and brain injury

Systematic and peripheral risk factors can increase the pathophysiological response to the stroke and brain injury such as neuroinflammation. Stroke occurs due the hypertension which may lead to the heart problems. Clostridium butyricum is a bacterial strain which cause neuroprotective in an animal model of cerebral ischemia reperfusion injury(Winek *et al.*, 2016).

Epilepsy, amyotrophic lateral sclerosis and Huntington disease

Researchers and clinicians are focused to recover the microbiota in gut for these diseases. Because if microbiomes of gut is regulated then physiology and behavior of these diseases become normal. According to the studies ketogenic diet is used for the treatment of epilepsy. Because it regulated the gut microbiota. Amyotrophic lateral sclerosis is caused by the lower abundance of butyrate producing bacteria. Huntington disease is occurring due to genetic disorders.Intrinsic factors (e.g., changes to protein homoeostasis, mitochondrial dysfunction, and uncontrolled corticostriatal input) and extrinsic environmental factors (eg, ethnicity, geographic region, tea consumption, and alcohol and tobacco use) can medium development of Huntington's disease.(Tremlett *et al.*, 2017).

Effect of Gut microbiomes on maternal and neonatal health

In the early life of neonatal microbiomes, there are different microbial species in neonatal gut that progressively stimulates and promotes gut maturation. A gut microbiome that is fully developed in neonatal stimulates the other health aspects and acts as a driver for stimulation these health factors. In neonates, the gut microbiome shows higher adaptability and plasticity than exhibited by the adults. It usually stimulates the long term health aspects in neonates and helps them to grow developed properly.(Rromano-Keeler *et al.*, 2014)Many important studies are emerging that describe the importance of gut microbiome during the early life as it supports the immune system of neonates. It also helps in host metabolism and colonization resistance to many other infectious agents. This relationship between the gut microbiome and immune system is a type of symbiotic relationship as it gives susceptibility to many other diseases later in life by providing strength to immune system in neonates (Tito *et al.*, 2012). In certain neonates, the gut microbiome is sometimes altered with metabolism uncovered the links as it increased susceptibility to the development of food allergy, asthma and autism.Many commensal bacterial species that are known as microbiomes effect on infant health and human diseases. Neonatal gut microbiomes are usually colonized with maternal and environmental flora and grow and get mature toward a stable composition over 2–3 years (Yatsunenko *et al.*, 2012).

Parental microbiome

Parental dietary intake changes the microbiome of offspring like the fat intake of parental microbiome dietary fatty acids also effects immunity, partially through modulation of responses to microbes**.** High fat intake in parental dietary fat consumption during gestation and lactation stimulates and strengthen offspring immunity. There was a study that was conducted on mouse models using previous observations in search for the influence of high fat intake in their dietary and their effect on the offspring immunity (Tappy and Le, 2010). They compare pups of mice fed either a Western diet fatty acid profile or a standard low-fat diet. They hypothesized that diet of parents are much effective on immunity of offspring certainly. So, Pups from western diet breeders were not diabetic or obese but still had bad results in models of infection, autoimmunity, and allergic sensitization. They showed colonic inflammatory responses with the increased amount of circulating bacterial LPS and muted systemic LPS responsiveness. These harmful impacts of the WD were associated with the changes of the offspring gut microbiome (Myles *et al.*, 2014)**.**

Effect of maternal gut

A continuous supply of nutrients and substances to the fetus is important for the fetal development and maturation during pregnancy. The need of getting a supply of nutrients and substances of a fetus from the mother changes with the gut microbiome of mother. There are many important metabolites of maternal gut that are originated from certain microbial species across the placental barrier and contribute to the formation of the blood-brain barrier and innate immune development in fetus. The nutritional status of mother also influences the placental function give alterations to the structure and metabolic potential of the maternal bacterial microbiome.(Jašarević et al., 2017)

Effects on vaginal microbiomes during pregnancy

During childbirth, the bacterial communities that are present in the maternal vagina also play a effective role in offspring postnatal growth and development. The bacterial communities in maternal vagina, provide the primary inoculum that take place in colony form in the neonate gut at birth, the composition of these pioneer communities contributes disproportionally to long-term health outcomes. The transmission of bacterial colonies that colonize the vagina has been proposed to make a disordered interaction of neonate-microbe that is necessary for immune education, metabolism, and neurodevelopment (Tremlett *et al.*, 2017)**.**

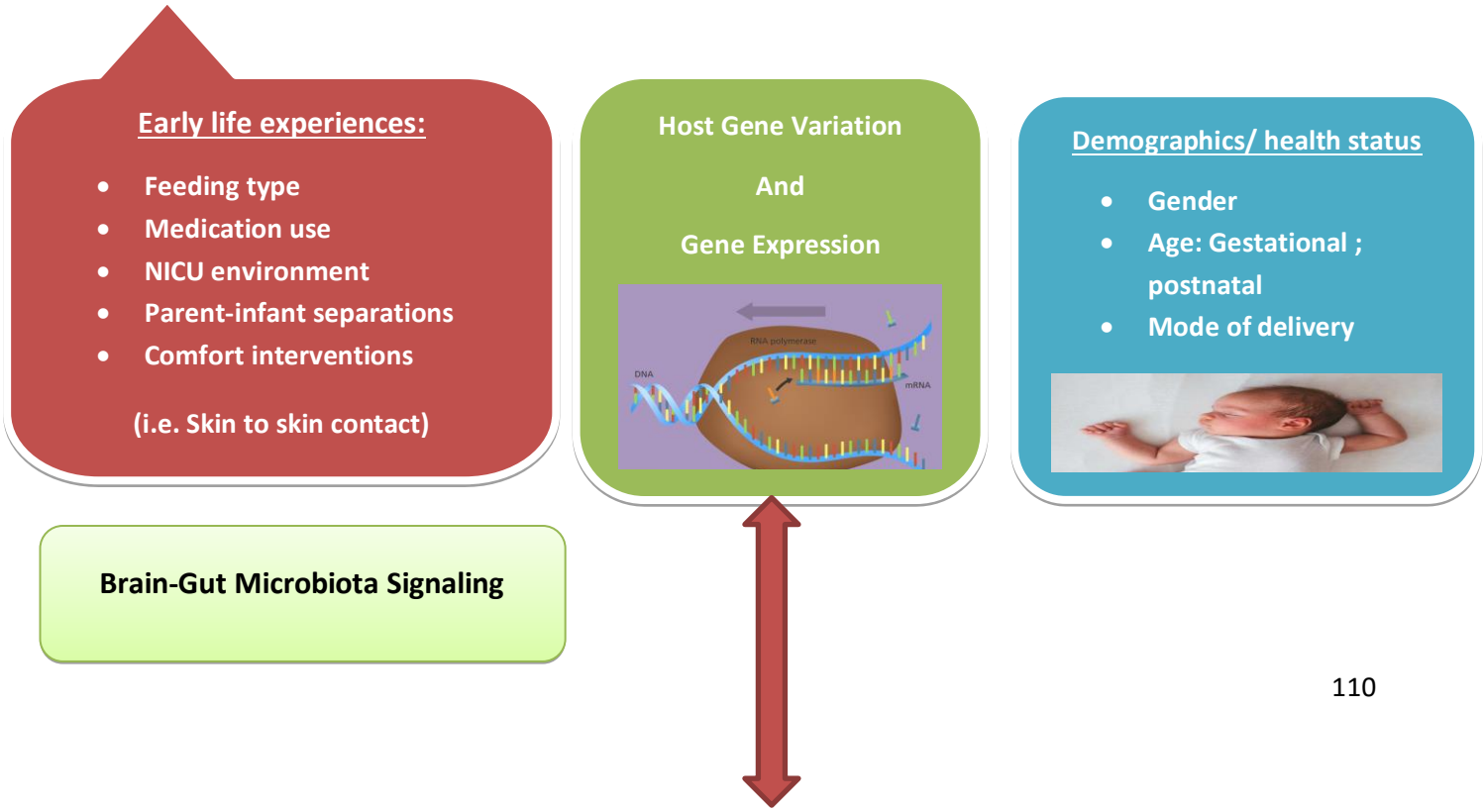
Effect on postnatal brain development

Gut microbiome plays a very bidirectional communicator role in between gastrointestinal tract and nervous system as it grows and develops throughout the lifespan of offspring (Fig 3). The development of gut microbiome in early life may lead to brain development procedures and it also effects the brain development processes afterwards in life like may cause brain disorders and other neuro-degenerative diseases in an individual (Winek *et al*., 2016).

The evolutionary trajectory of infant microbiome is related to process and growth of infant brain development. There are various factors that influence microbial gut community of individuals as it is a dynamic ecosystem for an infant. These factors include:Hospital environment, Use of medication, Delivery mode, Feeding, Life experiences, Gender, Gestational age and Postnatal ageThe gastrointestinal tract microbiota plays a significant role in the brain axis that include central nervous system, endocrine nervous system, autonomic nervous system evolving

sympathetic and parasympathetic nervous system, and also hypothalamus-adrenal pituitary axis. (Hantsoo *et al.*, 2019).

This signaling of whole network enables top-down communication from the brain to the neurons and secretory hormones of GI tract. In the bottom-down communication, signals influence from gut to affect the parts of brain like hypothalamus and amygdala that control stress and emotional activities of body (Rromano-Keeker *et al*., 2014) . So, these signaling networks can influence the alterations in brain like hypothalamus and amygdala that control stress and emotional activities of body. So, these signaling networks can influence the alterations in brain functions via GI tract.



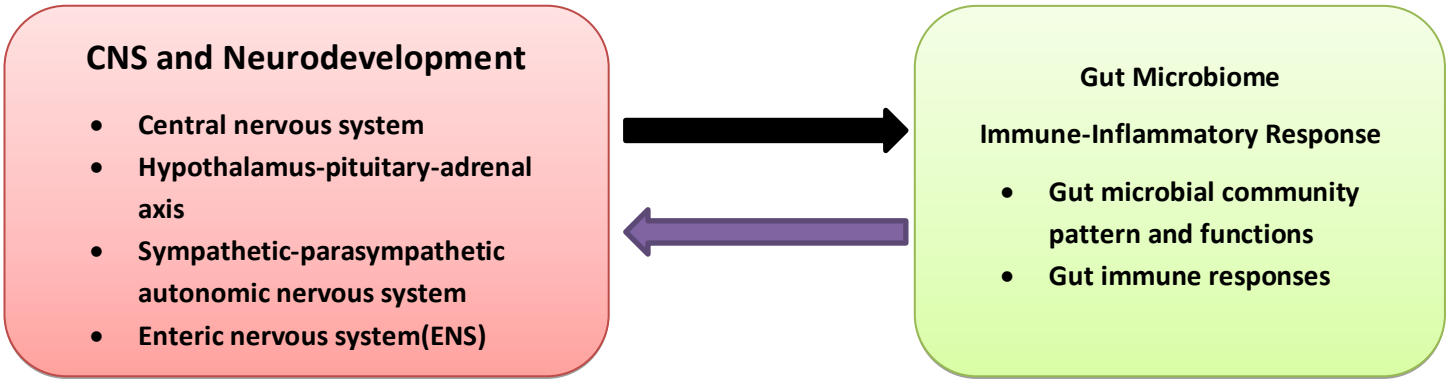


Figure3. showing the effect of gut microbiome on neonatal cognitive health

Gut microbiomes modification for improved cognitive; Diet

Diets that are high in fat and sucrose are mostly known as western diet. They play a significant role in influencing behavior and gut microbiome modifications. The diet that induces modifications in gut microbiome causes alterations in anxiety, cognitive flexibility and brain learning skills. Studies have shown that the diet that is high in sucrose can cause impaired spatial memory that is a loss in the brain functions and flexibility of cognitive health also get affected. So, diet plays a very major role in gut microbiome in changing the behavioral characteristics for cognitive health. Obesity is the usually associated with cognitive impairments that reduces quality of life and quality of health system (Collins *et al*., 2012). These impairments are important for response inhibition, episodic memory and spatial cognition related to brain. The hippocampus part of brain changes the food seeking behavior in humans that some people show a driving behavior in eating food quickly and take more meals per day than the actual requirement that later on leads toward obesity and other health issues (Ding *et al.*, 2010). This change in function of hippocampus works well in obesity and may impair adaptive decision making around eating and food. These impairments lead to cause changes in weight loss and cause the more weight gain in humans. The results of obesity in middle aged people usually compares with cognitive performances, cognitive decline risk and dementia problem in humans (Cryan *et al*.,2020). It can reduce volume of brain and changes the connectivity of white matter and volume of grey matter also reduces due to it. These changes in the brain structure usually

impair with cognitive health and behavioral characteristics and it depends on function of hippocampus and temporal lobe. The other research related to these diet related problems may lead towards improvement in cognition and thus also helps in quality of life for humans.(Davidson *et al*., 2018)**.**

Role of nutrition in obesity related to cognitive impairment_

The type of nutrition or diet is a major factor that is related to cognitive impairments or causing obesity and its effects on human health. The western diet is usually being discussed_ the diet that contain high amount of saturated fats along with added sugars that cause negative effects on cognitive health functions like alteration of hippocampus functions (Hasan Mohajeri *et al.*, 2018). The factors included in this diet are the influence of gut microbiome towards brain by using the axis between them that is gut-brain axis by changing the proportion of commensal bacteria in GI tract. So, the negative effect of consuming this diet impairs the neurocognitive functions that link the gut microbiome with dietary and metabolic impairment of hippocampus and associated mnemonic lacking.The gut barrier is a specialized semi-permeable and mucosal barrier strengthened by tight function proteins. This barrier allow nutrient and water entry and also prevents the entry of other harmful substances but western diet consumption allow the entry of these harmful substances as well. It causes changes in the function of brain parts that strongly impacts the neurocognitive functions and alteration in these functions causes impairment in cognition.The western diet consumption impairs the permeability of gut microbiome that allows the harmful chemical substances to enter into the microbiome and usually develops the metabolic disorders and also causes the neurocognitive impairment (Maffeis and Morandi, 2017).

The Gut Micro biomes and Immune System

As the mammals gut region contains microorganism's community which is biologically called as microbiota such as viruses, fungi, bacteria and parasites. Microbial genome consists of 3 x 10^6genes in their genetic sequences which in comparison to human genome is 15folds compact.The symbiotic relationship of the host and the microbe is mutually beneficial. The host in actual provides the microorganisms with basic nutrients and shelter and in return the Gut micro biota help in promoting the growth of metabolic system and maturation of intestinal immune system by providing useful nutrients such as vitamins and fatty acids. Microbial colonization actually occurs by the development of immune system. Fast and immediate

colonization of gut micro biota in the GIT tracts on new burns actually help in providing immunity by developing gut immune system.(Hantsoo *et al*., 2019).

As we know that immune system in actual is regulated by immune organs, immune cells, soluble cytokines and cell receptors. The intestines mucosal immune system is actually dependent on 3 main parts are like payer s Patches, Lamina propriety and the epithelial

As mucus layer of epithelial is considered as the first line of defense. The mucus layer and AMPs actually acts as mucosal barriers which help in restricting the symbiotic bacteria. Epithelial cells are considered as the 2nd line of defense in the intestinal mucosal immune system by either direct defensing or by sending chemical signals in the form of cytokines or chemokine's.The balancing of the intestinal immune system and gut micro biome plays a crucial role in maintaining the host homeostasis and defense.The dynamic interaction of the gut micro biome and environmental factors actually shape up the mucosal and systemic immunity. Diet and exogenous substrates are considered the most crucial parts of regulation of intestinal or gut micro biome.(Richards *et al*., 2016).

Gut microbiome Dysbiosis

The main aim of intestinal micro biota and mucosal immunity is to maintain intestinal homeostasis. As if the balance or homeostasis is disrupted it will lead to many major consequences such as IBD.

IBD

It is abbreviated as Inflammatory Bowel disease. It is actually result as the imbalance of homeostasis .It is heterogeneous disease which is influenced by a lot of factors such as genetics, environmental and microbial. This all results in the inflammation or destruction of intestines and an abnormal immune response. Crohn's disease and Ulcerative colitis are the major IBDs.Intestinal disposes lead to an abnormal immune system which in result cause inflammation or serious effects on the gastrointestinal tract. Although studies have shown that the Rapid formation of Next Generation sequencing technology which actually helped in identifying the causative specie for intestinal disposes.Gut micrbiota actually produced many immunogenicity substances such as Bacteriodes fragilis. As it play a crucial and very positive regulatory role in the human system. Intestinal microbiota modification acts as powerful preventive and therapeutic tool against inflammations.(Rromano-Keeler *et al*., 2014).

Intestinal permeability, immune system and systemic inflammation

Recent researchers have predicted that diet has strong influence over the intestinal micro biota and this ultimately affects the intestinal permeability and also cause inflammation. The dietary fluctuation like high fructose and hi fat diets on the gut micro biome.

High fat diets, As the high fat diets cause reasonable changes on the gut micro biome as it also effects on intestinal permeability , inflammation and other obesity related complications. As this high fat diet influenced the gut micro biota permeability and normal functioning which results in the metabolic endotoxin which is crucial for the production of metabolic syndrome. High fat diets have result in increment in the body weight and also cause ideal tumor necrosis factor as well. High fat diets have also caused alteration in gut epithelial integrity microbiota which cause systemic inflammation which in further results in obesity. Diet induced changes in microbiota causes the development of metabolic endotexmia, insulin resistance and metabolic syndromes. (Ding *et al.*, 2010).

High Fructose diets

High fructose diets cause hepatic and extra hepatic insulin resistance, obesity and insulin resistance and hypertension. Fructose is actually now a major part of the modern diet now. As fructose is considered the major culprit in promoting obesity.(Tappy and Le, 2010)

Systemic Inflammation

Interactions of micro biota, diet, gut permeability causing endotexmia and inflammation. Both high fat and high fructose diets because hepatic steattosis. Hepatic inflammation can cause predispose of other organs such as lung, kidney and brain. Intestinal permeability allows gut derived toxins cross the intestinal barrier and activates the Kupffer cells which are primed to produce inflammatory cytokines which cause liver inflammation or injury and systemic inflammation. There are gut derived products which actually gut derived toxins crosses the gut barrier which are not only endotoxins but LPS.

Cognitive dysfunction

There is this possibility of alteration in brain physiology and behavior by changes in gut microbiota. As in old studies cognition is always linked with brain and central nervous system organs. So now non-nervous system factors also influences in the cognitive dysfunctions such neurodegenerative diseases, cerebrovascular diseases by gut resident bacteria of GIT tract. Changes of blood brain barriers ,brain vascular physiology and brain structure are among the

critical reasons for the brain dysfunctions. The habitat of microbiota do have some influence too as the bi-directional brain-gut signaling through humeral ,neural and immunological pathogenic pathways.The CNS changes the gut microenvironment by maintaining gut motility and secretion and mucosal immunity by the help of neuronal glial epithelial pathways. Bacteria react to the changes caused by the extrinsic and intrinsic factors by the formation of neurotransmitters or neuromodulators in the intestine to effect host CNS.(Collins *et al*., 2012)

White adipose tissues and Leptin concentrations

Imbalance accumulation of white adipose tissues in overweight and obese patients generally occurs as a result of increase the circulation levels of adipokines. Adipose dysfunction and adipokine deregulations are considered to be the culprits in promotion of obesity and other obesity related disorders. So any increase in the adipose tissues leads to obesity which in turn effects the CNS which in turn altered the brain metabolism , brain atrophy , neuron inflammation and neuronal dysfunction , mood and behavioral changes and cognitive declines.It is now cleared that adipose tissues do effect the cognitive dysfunctions (Krabbe *et al*., 2004).The adipokines do effect the brain morphologies.Adipose tissues form adipokines type leptin and TNF-a actually are involved as they can cross the blood brain barriers and directly act on brain while other act on brains epithelial cells.so in result they cause many pathological changes in the brain functions such as inflammations , blood brain barrier integrity is also compromised (Myles *et al.*, 2014).

Leptin Concentrations

It is majority produced by Adipose tissues specifically by subcutaneous tissues in humans and by white adipose tissues in rodents. Circulation of leptin influenced by multi factors such as metabolism , body fat weightage , sexual dimorphism and circadian rhythms. Leptin actually cross the blood brain barrier by getting bind to the leptin receptors or getting interacted with them .

Leptin Resistance

Response to leptin also decreases with obesity, aging and neurodegenerative disease in a process called as Leptin resistance. Leptin resistance effects a lot of processes such as food intake , insulin sensitivity ,inflammation and cognition. Leptin resistance actually promotes the production of leptin by adipocytes and hyperleptinemia. Triglycerides also cause hindrance in

blood brain barrier leptin transport which in result causes central leptin deficiency. (Forny-Germano *et al*., 2019).

Discussions and Results

Peripheral inflammation signaling in humans

To interpret the changes in the CNS the way of communicating must be among CNS and periphery. As fever and neuroendocrine pathologies have depicted that periphery communicate to CNS through by neural or humeral pathways.Peripheral inflammatory mediators have impacted on brain function and they also play crucial roles which cause inflammation and other symptoms like other cognitive disorders. As the systemic infusion of inter leukins and tumor necrosis factors alpha also promotion suppression food intake , poor memory ,social exploration. It also cause cognitive dysfunction in peripheral lipopolysaccharides and endotoxins which promote the stimulation of proinflammatory cytokines (Jackson *et al*., 2018) . This interferes among many neuromolecular processes like hippocampal neurogenesis, synaptic plasticity and synaptic scaling as it cause dendritic atrophy which ultimately effects memory and thought. Aging has also been related with proinflammatory cytokine expression in the periphery (Krabbe *et al*., 2004).

Central Inflammation signaling

Many epidemiological researches are giving importance to the role of inflammation in the modeling or affecting the cognitive function. The gut receives regulatory signals from the CNS. This bidirectional interaction has some serious effects. The gut-CNS signaling occurs through the central regulation satiety. Diet patterns as they are controlled by CNS food intake which ultimately affect the nutrient availability to the gut. Satiation signaling peptides are the key modulators which help in CNS-Gut control. These signals are usually raised from the GI tract and sometimes from the brain too.CNS can effect gut micro biome by both pathways neural and endocrine pathways either by direct and indirect ways.CNS disorders are either considered as immune mediated and non immune mediated. Immune mediated disease are actually triggered by autoimmune disorders such as multiple sclerosis and Non immune mediated disorders are stress , depression , anxiety and autism.(Ochoa-Repáraz *et al*., 2011).

Blood Brain Barrier

Gut micro biota is considered as one of the pivotal member for the blood brain integrity. As BBB is actually a semipermeable barrier which is made up of specialized epithelial cells in the microvasculature.It also differentiate the CNS from Peripheral blood.Microorganism induced blood brain barrier dysfunction cause many psychological disorder such as stress, anxiety, depression and autism spectrum disorders. And many critical neurological disorders such as Parkinson's disease, Alzheimer's disease and Schizophrenia.Possible way by which micro biota may have affected the cognitive. Health are Either BBB modulation by the gut influenced neurotransmitters and bacterial metabolites. May metabolic diseases such as Diabetes in which the blood brain barrierPermeability is increased. This all lead to Alzheimer's disease amyloid b peptidedeposition.Microbiota disposes also caused disruption in theProtective function of blood brain barrier such as its permeability, behavioral changes and tightjunction changes (Mouries *et al*., 2019).

Gut Micro biome and Psychiatrist perspective

Imbalances of gut micro biome have serious effects on brain and it cause serious psychiatric illness.The role of micro biota in regulating mood cognitions, stress, anxiety and social behaviors. The role of micro biome in causing psychiatric disorders by causing alteration in theDiversity of the Disorders in treatment of autism, schizophrenia and attention deficit autismsome mood disorders such as bipolar , stress and anxiety disorders.Microbes in gut are suspected to alteration by treatments or medication used to treat

Psychiatric Disorders so they get to play important roles in maintaining the gut metabolism either by naturally or orally administered medication.The beneficial

Strategy of micro biotas to be used as treatment technique is as we know micro biotas are Readily diverse and dynamic so micro biota are manipulated by number of factor such as food, diet exercise and stress reduction.(Breit *et al*., 2018).

A quote from Hippocrates, "Let food be thy medicine and medicine be thy food,"

So personalized medication are being used according to the nature of gut micro biome as a Base for The treatment of psychiatric disorders and future adjunct therapies.

CONCLUSION

Reviewed that gut microbiomes (GMB) linked with brain through vagus nerve and by producing neurotransmitters such as GABA, acetylcholine and serotonin. It not only help to modify human physiology such as CNS functions and cognition but also experimental animals show that GMB dysbiosis causes negative effects on cognitive functions, can be treated by the prebiotic, probiotics and dietary modifications. Further researches are required to identify of pathology of microbiomes and individual changes to the microbiomes pattern. Many external and internal factors also affect the microbiota and it can be modified by diet and other intervention. Clinical studies proved that gut microbiomes may affect the nervous system and causes neurological disorders. Neonatal gut microbiomes are usually colonized with maternal and environmental flora and grow and get mature toward a stable composition over 2–3 years. The gastrointestinal tract microbiota plays a significant role in the brain axis that includes central nervous system. The diet that contain high amount of saturated fats along with added sugars that cause negative effects on cognitive health functions like alteration of hippocampus functions. Gut micro biota is crucial for the Intestinal homeostasis and for the brain health. The Gut micro biota have great impact on the immune System of The human as any alteration to the gut microtome will lead to serious ailments such As obesity, diabetes and many other neurodegenerative ailments. However there are still some Modifications required in the study and analysis of experimental design, subjects, and Models. Analytical approaches and quality control protocols in the study of gut micro biome in Relation to cognition. As the relation of host with symbiotic microorganism is considered as Clear Association rather than a casual interaction. So diet, lifestyle approach genetic and Environmental factors have serious influenced over the modifications of demographics of gut microbiome in relation to cognitive health.

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ABSTRACT

Cellulosic organic wastes like wheat, rice, cotton straws and corn stoves are being utilized as feeds of animals since past history. However, ample amount of cellulosic materials are being wastes every year and not being utilized for generation of economical resources.

Second generation biomass in Pakistan and some neighbor countries are just putting on fire to clean up land for cultivation of next seasonal crops which is adversely affecting the our environment. .A study was conducted for the production of bioethanol using wheat , cotton and rice straws, corn stove as well as peel wastes . The experimental was conducted by assessing various steps like acidic/ alkali pretreatment, cellulases hydrolysis along with effect of acid proteases on cellulose degradation as well as yeast fermentation processes . Results indicates that higher level of ethanol was produced depending upon on substrates concentration , optimized condition of fermentation. Higher level of glucose (g/L) was obtained from by using acid treatment and higher amount of ethanol was obtained from after during fermentation process . It was observed that use of acid protease enhanced final recovery of ethanol. It is expected that outcome of this study will help to increase production of biofuels to reduce burden of foreigner exchange that is currently being utilize to import fossil fuel from other countries.

Key words; Organic wastes , Polysaccharides , Fermentation, Bio fuels

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INTRODUCTION

Fossil fuels, including coal, oil and natural gas, are currently the world's primary energy source. Formed from organic material over the course of millions of years. When fossil fuels are burned, they release carbon dioxide and other greenhouse gases, which in turn trap heat in our atmosphere, making them the primary contributors to global warming and climate change (Galbe and Zacchi, 2007).

Biofuel, any fuel that is derived from biomass—that is, plant or algae material or animal waste

Biomass is a vital energy source in Pakistan because of agriculturally based country. The biomass produced in livestock and agriculture sector in the form of animal waste and crop remaining as sugarcane bagasse and rice husk (Ariffin *et al.*, 2006; Chaudhry*et al*., 2009). Second generation biomass is mainly composed of lignocellulosic material. Lignocellulosic biomass is more plentiful organic substance on earth and consists of cellulose (35-50%), hemicellulose (20-35%) and lignin (5-30%) (Becerra *et al*., 2015). Various renewable energy resources include different agricultural substances like green leaves, fruit shells, straws, nut shells and fruit seeds (Bergey *et al.*,1994). Most commonly used feed stocks are wheat straw, wheat bran, corn stover, corn steep liquor and apple pomace (Breznak and Brune,1994). Now a day, agricultural waste is used to produce biofuels like biodiesel, bioethanol, biohydrogen and methane as compared to energy crops because they have competition with food crops. As huge amount of agrowaste is available and have discarding problem so, alternate option is the utilization of lignocellulosic biomass in order to reduce the competition between fuel and food . The grasses are considered as reliable substance for extraction of ethanol. The utilization of perennial grasses is advantageous and possibly it further decreases the cost for the production of ethanol and its use as fuel (Brune *et al*., 1995).

Exploration of various sources for alternate energy have been increased because of increasing concerns about energy security and climatic changes. The transportation sector plays a significant role for emission of greenhouse gases due to uses of fossil fuels , However, replacement of oil derived fuels such as ethanol could reduce environmental impacts and give advantages on social as well as economical levels (Chaudhry *et al*., 2009). Various alternatives steps to generate sustainable biofuels from biomass has been investigated. Important biological energy resources are like bioelectricity, biogases, biodiesel and bioalcohols. Among these sources, bioalcohol shows a great potential to reduce the emission of greenhouse gases, decrease the dependence on fossil fuel and act as potential fuel for transport sector (Dheeran *et al*., 2012). The production of bioethanol has been improved extremely because many countries are trying to reduce the import of oil, improving the quality of air and growing rural economics. The global ethanol production is 51,000 million liters (Galbe and Zacchi, 2007; Iram *et al*., 2021). Ethyl alcohol has some advantages as a fuel as it has higher oxygen contents. The higher oxygen level permits improved oxidation of hydrocarbons with successive reduction in aromatic compounds and carbon monoxide emission. While ethanol has greater octane rating properties (Lehman, 2005).

The simultaneous saccharification and fermentation process combines polysaccharide hydrolysis and fermentation in one step, but still relies on the addition of exogenously produced enzymes. In addition to pretreatment and addition of exogenously enzymes, the major rate limiting step in enzymatic hydrolysis is the protease attack that reduces the enzyme activity, especially cellulases. The simultaneous saccharification and fermentation that occurs in this type of process is an attractive method for keeping monomeric sugars at low enough concentrations to avoid enzyme inhibition, thus reducing costs by decreasing the amount of enzyme needed for the process (Lynd *et al*., 2008).

Converting lignocellulosic biomass to ethanol involves four stages: pretreatment, hydrolysis, fermentation, and ethanol recovery by distillation. Pretreatment increases biomass digestibility for efficient fermentable sugar production, which reduces the cost of bioethanol production . Various pretreatment methods have been suggested, depending on the purpose of removing hemicellulos. or lignin from the biomass Dilute acid pretreatment is a promising pretreatment capable of high solubilization of hemicellulose This process degrades most of the hydrogen bonds in hemicelluloses and partially degrades cellulose and lignin (Mahon *et al*., 2011).

In addition, acid pretreatment permits hemicellulose hydrolysis of pentoses and hexoses, removes some of the lignin, and makes the cellulose structure more accessible, so that a fraction can be converted to glucose enzymatically.. The choice of pretreatment technology for a particular raw material depends on several factors, some of them directly related to the enzymatic hydrolysis step such as sugar-release patterns and enzymes employed. Thus, the combination of the composition of the substrate in addition to the pretreatment conditions has a great influence on biomass digestibility (Reiner, 2010)

A yeast is a unicellular fungus which reproduces asexually by budding or division, especially the genus *Saccharomyces* which is important in food fermentations (Rogers, 2008). Most yeasts are larger than most bacteria and their participation are importance in the food industry.

Saccharomyces cerevisiae (commonly known as baker's yeast) is a single-celled eukaryote that is frequently used in scientific research. *S. cerevisiae* is an attractive model organism due to the fact that its genome has been sequenced, its genetics are easily manipulated, and it is very easy to maintain in the lab. Most yeasts require an abundance of oxygen for growth, therefore by controlling the supply of oxygen, their growth can be checked. In addition to oxygen, they require a basic substrate such as sugar. Some yeasts can ferment sugars to alcohol and carbon dioxide in the absence of air but require oxygen for growth. They produce ethyl alcohol and carbon dioxide from simple sugars such as glucose and fructose.Yeasts are active in a very broad temperature range - from 0 to 50° C, with an optimum temperature range of 20° to 30° C.

The optimum pH for most micro-organisms is near the neutral point (pH 7.0) and are usually acid tolerant. Yeasts can grow in a pH range of 4 to 4.5 (Scharf and Boucias, 2010)

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Although C. thermocellum is a proven industrial ethanol producer in traditional starch-based processes, it will be no easy task to provide this microorganism with the ability to convert lignocellulosic biomass to ethanol. The carbohydrate components of lignocellulose (cellulose and hemicellulose) are tightly bound to lignin, making the sugars largely inaccessible to enzymes. Before enzymatic hydrolysis, pretreatment with acid or alkali and increase activity of

cellualses by action of protease inhibitors that reduce the activity of proteases is generally needed to fully maximize the release of sugars from any lignocellulosic biomass

Keeping in view importance of biofuels study was conducted with aims and objective like (i) Treatment of lignocellulosic biomass to break down into cellulose, hemicellulose and lignin by using acid/ alkali treatment .(ii) Optimization of various condition for yeast and bacterial fermentation process (iii) Production of higher yields ethanol by using cellulase and acid protease.

MATERIAL AND METHODS

Collection of Agricultural Substrates

Various samples (wheat, rice, and cotton straws as well and corn stover wastes) were collected from different areas of Punjab. The samples were dried, grinded passed through 40 mesh standard size sieve.

Analysis of Biomass Samples

All samples were analyzed for moister, ash, dry matter, crude protein, crude fiber, crude fat as well as dry weight contents were determined (AOAC, 1990). Cellulose, hemicellulose and lignin contents were quantified by using standard method described by many authors including Scharf and Tartar (2008).

Acidic and Alkaline Pretreatment of biomass samples

Pretreatment process was performed by using H_2SO_4 and NaOH (1.0, 1.5 and 2%) at diverse temperatures such as 100 °C , 110 °C and 120 °C for different times durations (15, 30, and 45 minutes). Solid sample (10 %) (w/v) in reagent bottle was utilized during experiment. After

pretreatment, the vacuum filtration assembly was used for filtration of samples in each bottle and the contents were emptied on filter paper. After filtration, the solid was wash away with 300 ml distilled water to neutralize the pH and filter paper was than dried at 105 ℃ and weighed.

Enzymatic Hydrolysis

The biomass samples after pretreatment 5% (w/v) was hydrolyzed with cellulase and β-glucosidases at 50 °C and 160 rpm for 72 hours in a water bath shaker with 0.05 M buffer (sodium citrate) at 4.8 pH. Chloromphenicol (100 µg/ml) and ampicilin (100 µg/ml) were also added during reaction to inhibit microbial growth. Cellulases from *T. reesei,*cellobiase from *Aspergillusniger* and Novozyme 188 was delivered by Novozyme A/S, Bagsvaerd, Denmark having activity of (30FPU g-1). The samples were withdrawn from reagent bottle after every 12 hours to determine the concentration of sugar (Shields and Cathcart, 2010).

Acid protease .A protein-digesting enzyme that exhibits maximum activity and stability in acid conditions (pH 2.0–5.0) and is inactivated at pH values above 6.0. Acid protease are helpful in food and wine industries for extraction of higher yields of ethanol. Therefore in current study addition of cellulase was supplemented with acid proteases to increase yield of ethanol

After enzymatic hydrolysis, H_2SO_4 (µl) or NaOH was added. Un-hydrolyzed sample was separated by centrifuging for 10 minutes at 13,500 g. Supernatant was collected by means of syringe filters.The amount of sugar was determined by p-hydroxybenzoic acid hydrazide (PAHBAH) method against standard curve(1Mm-25mM of xylose.The best pretreatment condition was selected after enzymatic hydrolysis process. The sample containing higher amount

of released sugar was further selected for fermentation process and solid biomass was stored at 4 °C.

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Saccharification

The agro and municipal waste samples (wheat, cotton , rice straws and corn stover wastes) were taken as a solid loading of 5% (w/v) and then autoclaved. The crude enzymes from bacterial species were added and the ratio of substrate to enzyme was adjusted to 1:1 and placed for 72 hours at 50^{o}C. Both of the enzymes were added in separate reaction mixture in order to check the individual enzymatic activity. Also both enzymes were mixed at a ratio of 1:1 to check the combine effect of enzymes and after scarification, the sugar contents were determined (Tokud and Watanabe, 2007).

Culture conditions for growth

Saccharomyces cerevisiae strain was maintained on YPD (yeast extract 1% (w/v), peptone 2% (w/v) and glucose 2% (w/v)] agar medium at 4°C. Culturing of yeast cells was carried out in a 5-mL tube of YPD medium containing NaCl 0.9% (w/v) at 30°C for 16 h on a rotary shaker (100 r.p.m.) according to Alfenore *et al*. (2002).

Separate Hydrolysis and Fermentation

Fermentation experiment was carried out by using *C. thermocellum* grown in glucose yeast extract broth medium for 48 hours and 10% inoculum was inoculated into 50 mL fermentation medium containing previously saccharified solution and kept for 3 days at room temperature Fermentation experiment was performed at 50^{0}C and 120 rpm for 72 hours under anaerobic conditions. After completion of fermentation reaction, the obtained mixture contains methanol, butanol, ethanol and acetone were removed by fractional distillation process in a fractional

distillation apparatus on the basis of boiling point. As butanol has higher boiling point (118 ^{0}C) than water (100 ^{0}C) Butanol can be condensed then separated. The boiling point of ethanol is lower (78.3 ^{0}C) in comparison with water that's why it can be condensed earlier than water (Watanabe *et al.*, 1998).

HPLC Analysis of Enzymatic Hydrolysate

The fermentation products like monomer sugars (hexoses and pentoses) acetone- butanoland ethanol as well as others bio products were determined by using method reported by Wenzel *et al*. (2002).

The enzymatically hydrolyzed samples of acidic and alkaline pretreatment of wheat and rice straws as well as corn stover were further analyzed by HPLC. For this purpose, the samples those have shown higher amount of glucose at optimized conditions were used for analysis. The samples those were withdrawn at different time periods during enzymatic hydrolysis, then these were centrifuged at 14,000 rpm, at 4 ºC for 15 minutes. Supernatant was separated and then filtered by using 0.22 µm syringe filter. An aliquot of the sample (500 µl) was diluted with 1ml methanol to bring the concentrations of the samples within the range of calibration curve. Methanol was used due to the solubility of the sugars. All the samples and standard solution of glucose was passed through the 0.22 µm filter prior to analysis. About 20 µl of agrowaste sample was injected through injection loop into HPLC system. In order to analyze the glucose, enzymatically hydrolyzed samples were run in the gradient mode for 10 minutes (Shields and Cathcart,2010).

Statistical analysis

Data generated through various analysis were statically analyzed for mean, standard deviation etc.,

RESULTS AND DISCUSSION

Results regarding Physical and chemical analysis of biomass samples, pretreatment, enzymatic as well as quantification of end products by using High performance chromatography (HPLC) technique..

Proximate Analysis of various Biomass samples

Various samples of biomass were analyzed to get concentration level of dry matter, moisture, crude protein, lipid ash and fiber contents (Table 1). These parameters play important role to maintain quality of feed stock uses for different purposes.

Table 1. **Proximate analysis (%) of biomass samples**

Parameters	**Cotton stalks**	**Corn stover**	**Wheat straw**	**Rice straw**
Dry Matter	92.5 ± 2.6	89.8± 3.1	91.4± 1. 5	90.5± 0.5
Moisture contents	6.7± 0.8	7.5 ±0.9	7.8± 0.6	5.7± 0.6
Volatile Matter	77.6 ± 1.2	75.8± 2.6	89.3±2.5	90.6± 1.5
Fixed Carbon Content	17.5±1.2	19.5± 2.3	18.7± 1.3	17.5± 1.6
Ash Contents	8.7± 0.5	6.2± 0.4	5.1± 0.7	3.4± 0.6
Crude Fat Content	3.5± 0.2	3.7 ± 0.8	3.6±0.3	2.8± 0.4
Crude Protein Content	4.2 ± 0.6	6.8 ± 0.9	9.6± 0.4	4.7± 0.5
Cellulose Content	37.5±1.5	33.6±2.1	38.5± 2.7	34.8± 1.7
Hemicellulose Content	28.5± 2.5	26.5± 2.8	27.8 ± 3.1	26.7± 2.8

Lignin Content	14.8± 2.6	19.5± 1.5	13.7± 2.4	15.8± 1.9

Pretreatment of Agricultural substrates

Samples of wheat, cotton, rice (straws) and corn stover wastes were used for pretreatment process. Maximum amount of sugar (17.5 ±1.6mM/l) was found in wheat straw, when sample was treated with H_2SO_4 . At a concentration of 3 % very less amount of sugar was detected 6.38±0.86 mM/l (Table 2). Probably amount of released sugar may be converted into inhibitors such as hydroxymethyl furfural and similar others products (Garcia et al., 2011). During alkali pretreatment higher amount of sugar (16.5 ±0.1mM/l) was released when sample was treated with NaOH (3%)(Table 3) . In cotton stalk, higher amount of sugar (14.2 ±0.03mM/l) was obtained when 1 % H_2SO_4 was used.When sample was treated with 3 % NaOH the sugar concentration and acidic pretreatment of rice straw higher amount of released sugar all values are mentioned in table 2. It was observed that values of sugar obtained by two different treatments are according to results reported by other authors.

Table2. Chemical pretreatment of biomass samples with different concentrations (%) of H_2SO_4 release of sugars (%), after 72 h duration

H_2SO_4 (%)	Temp (C)	Time (min)	Wheat Straw	Rice Straw	Cotton stalk	Corn stover
1	105	10	11.9± 0.3	7.8 ± 0.5	6.7 ± 0.8	14.9 ± 0.4
		15	11.8± 0.4	9.8 ± 0.4	9.2 ± 0.5	15.2 ± 0.7
		20	11.5± 0.8	8.5 ± 1.5	9.8 ± 0.6	15.1 ± 0.7
	115	10	8.6± 0.5	11.5 ± 0.4	10.3 ± 0.5	13.8 ± 0.5
		15	7.9± 0.2	12.8 ± 0.3	11.7 ± 0.3	13.9 ± 0.4
		20	8.1± 0,7	9.5 ± 0.1	9.8 ± 0.1	10.8 ± 0.7
	125	10	12.1± 0.7	13.8 ± 0.2	10.8 ± 0.2	14.9 ± 0.1
		15	12.7± 0.8	14.2 ± 0.4	12.90 ± 0.1	14.8 ± 0.1
		20	15.6± 0.7	9.1 ± 0.7	15.01 ± 0.5	15.1 ± 0.3
1.5	105	10	11.2± 0.7	15.3 ± 0.6	12.6 ± 0.1	14.9 ± 0.1
		15	11.9± 0.5	13.5 ± 0.6	13.1 ± 0.4	14.2 ± 0.4
		20	9.5± 0.6	12.0 ± 0.8	13.5 ± 0.7	15.1 ± 0.3
	115	10	11.6 ± 0.8	12.8 ± 0.6	13.0 ± 0.2	13.2 ± 0.0
		15	9.5±0.3	16.2 ± 0.5	9.1 ± 0.5	9.2 ± 0.1
		20	12.5 ±0.7	14.6 ± 0.1	10.6 ± 0.2	16.6 ± 0.7
	125	10	14.6±0.8	11.2 ± 0.6	14.1 ± 0.6	14.8 ± 0.5
		15	16.5±0.9	12.8 ± 0.7	9.1 ± 0.2	15.9 ± 0.2
		20	15.6±0.6	11.6 ± 0.2	10.5 ± 1.0	16.1 ± 0.9

2.0	105	10	13.2±0.5	13.2 ± 0.6	14.2 ± 0.4	15.1 ± 0.6
		15	14.3±0.7	11.9 ± 0.5	12.2 ± 0.6	14.0 ± 0.7
		20	12.6±0.9	11.3 ± 0.6	**16.4 ± 1.0**	**17.1 ± 0.9**
	115	10	12.3±0.8	16.1 ± 0.2	12.2 ± 0.4	15.1 ± 0.1
		15	13.7±0.5	12.2 ± 0.7	12.6 ± 0.5	15.2 ± 0.6
		20	14.5±0.6	16.4 ± 1.0	13.1 ± 0.8	14.9 ± 0.5
	125	10	14.6±0.3	14.0 ± 0.3	12.8 ± 0.7	15.1 ± 0.2
		15	16.7±0.8	15.1 ± 0.1	14.2 ± 0.5	14.8 ± 0.1
		20	**17.5±1.6**	**16.5 ± 0.1**	14.2±0.3	15.6 ± 0.1

Chemical treatment of biomass samples for sugar . Mean ± ST

Table3. Chemical pretreatment of biomass samples with different concentrations (%) of NaOH to release of sugars (%).(g/L) after 72 h duration.

NaOH (%)	Temp (C)	Time (min)	Wheat Straw	Rice Straw	Cotton stalk	Corn stover
1	105	10	12.5± 0.4	5.2 ± 0.7	3.7 ± 0.6	4.9 ± 0.4
		15	12.8± 0.3	9.01 ± 0.1	5.2 ± 0.5	5.6 ± 0.4
		20	13.6± 0.8	11.5 ± 1.3	10.5 ± 0.2	11.1 ± 0.1
	115	10	8.7± 0.4	4.5 ± 0.9	13.1 ± 0.1	12.1 ± 0.3
		15	7.9± 0.2	5.8 ± 0.3	13.7 ± 0.3	12.9 ± 0.4
		20	8.1± 0,6	9.5 ± 0.1	9.8 ± 0.1	9.8 ± 0.7
	125	10	10.6± 0.7	13.2 ± 0.2	12.8 ± 0.2	13.1 ± 0.1
		15	12.6± 0.8	14.2 ± 0.4	13.90 ± 0.1	14.8 ± 0.1

		20	16.3± 0.5	9.1 ± 0.7	15.01 ± 0.5	12.1 ± 0.3
1.5	105	10	13.6 ± 0.7	12.8 ± 0.1	13.6 ± 0.1	12.9 ± 0.1
		15	15.5± 0.5	14.5 ± 0.1	14.01 ± 0.4	13.2 ± 0.4
		20	16.8± 0.9	15.0 ± 0.3	15.5 ± 0.4	15.1 ± 0.3
	115	10	11.6 ± 0.8	12.5 ± 0.1	13.0 ± 0.2	13.2 ± 0.0
		15	15.5±0.3	13.2 ± 0.5	9.1 ± 0.5	9.2 ± 0.1
		20	17.5 ±0.7	14.6 ± 0.1	10.6 ± 0.2	10.6 ± 0.2
	125	10	13.6±0.8	11.2 ± 0.0	14.1 ± 0.6	13.8 ± 0.5
		15	14.5±0.9	9.8 ± 0.1	9.1 ± 0.2	8.9 ± 0.2
		20	17.6±0.6	11.6 ± 0.2	10.5 ± 1.0	10.1 ± 0.9
2.0	105	10	13.5±0.8	14.2 ± 0.5	14.2 ± 0.4	13.9 ± 0.5
		15	14.6±0.4	8.9 ± 0.1	12.2 ± 0.6	11.0 ± 0.8
		20	15.3±0.5	10.3 ± 1.0	**16.4 ± 1.0**	**15.1 ± 0.9**
	115	10	14.2±0.6	14.1 ± 0.2	13.2 ± 0.3	12.1 ± 0.1
		15	15.8±0.9	12.2 ± 0.7	13.6 ± 0.1	12.2 ± 0.1
		20	16.2±0.7	**15.4 ± 1.0**	12.1 ± 0.2	11.9 ± 0.1
	125	10	16.6±0.5	13.0 ± 0.3	10.8 ± 0.3	10.1 ± 0.2
		15	18.7±0.9	13.1 ± 0.1	13.2 ± 0.2	12.8 ± 0.1
		20	**21.5±1.2**	12.1 ± 0.1	12.3+0.3	11.01 ± 0.1

Chemical treatment of biomass samples for sugar . Mean ± ST

Comparative study of treatments by using various substrates

It was observed that higher amount of sugar was produced when wheat straw was treated with Enzymescellulase and acid protease . It was not noted that amount of sugar released in both chemical treatments depends on natural of substrates used for analysis . All agro and municipal wastes substrates used in study contain reliable amount of sugars **(** Tables 2-3**).** Which is good indicator for production of ethanol on commercial scales and similar results are also reported by

Zhao et al. (2012)

The solid fraction of samples has given larger quantity of glucose when it was treated with dilute acid concentration for 30 minutes and the temperature of reaction was maintained at 110 °C. It proved that moderate temperature and acid concentration play key role to enhance the glucose contents during pretreatment. Similar finding on acid hydrolysis of orange peel at low temperature has been reported by Talo et al. (2014).

The reason behind higher saccharification (80.54%) was achieved as there was no accumulation of sugar like cellobiose occurred although cellobiose was available in reaction mixture (Williams , 2009)has also pointed out that the performance of celllases was actually enhanced (due to absence of cellubioses), and the results in higher sugar recovery after enzymatic hydrolysis (Yoon *et al*., 2007).

Saccharification of biomass samples with enzymes

Saccharification process of various biomass samples was carried out after acid/ alkali treatment with cellulase and acid proteases . Results indicates that wheat straw has released glucose followed by rice straw, corn , cotton straws and peel wastes (table 2-3). This released sugar, can than further be used for fermentation experiments.

Analysis of Sugar after Pretreatment and Enzymatic Hydrolysis

As the timeperiod increases, glucose concentration was reduced but ethanol concentration was enhanced but up to certain time limit. However, after 72 hours glucose concentration was not sufficient to maintain theethanol production. Higher cellulosic but lower lignin contents of

cogon grass was compared to Peel wastes and it was found that these contents make cogongrass a better candidate for ethanol production.

Spectrophotometric analysis and Comparison of sugar production in three agrowaste samples

Better glucose yields were obtained from wheat straw in all experiments after 72 hours of enzymatic hydrolysis (Table 4). It was observed during experiment that by increasing the concentration of H_2SO_4 from 0.5 to 1.5% the amount of sugar was also increased. In all experiments, higher yield of glucose was also recorded at a retention time of 20 minutes rather than 10 and 15 minutes. For acidic pretreatment conditions of wheat straw, the conditions were optimized at 120 ^{0}C, 20 minutes of retention time with 1.5% of sulphuric acid .At this concentration, glucose concentration was at peak. During alkali pretreatment conditions, the glucose yield was increased by increasing the temperature and higher yield was recorded at 120 ^{0}C. Meanwhile at similar temperature high yield of glucose was recorded when 1.5 % concentration of NaOH was used . By increasing the time of enzymatic hydrolysis from 0 to 48 hours sugar yield was increased but when the time is increased further to 72 hours sugar concentration was chopped.The decrease in glucose concentration was probably due to production of inhibitors by higher acid concentrations. Maximum reducing sugars (7.73 g/L) were obtained at 120 ^{o}C , when 1% NaOH concentration and reaction time of 15 minutes were used .Rice straw has shown higher glucose yield in acidic pretreatment conditions at 110 ^{o}C, acid concentration (1.5%) and retention time 10 minutes was used .The optimum condition used for rice straw analysis in case of alkaline pretreatment, temperature (100 ^{o}C), sodium

hydroxide concentration (0.5%) and retention time (20 minutes) . Higher yield was obtained after 72hours of enzymatic hydrolysis. During acidic pretreatment high yield of glucose was obtained at a temperature (120°C), H_2SO_4 concentration

Table 4. Products obtained after fermentation process

Substrate	Total concentration (g/L)	Total theoretical yield of ethanol (g/L)	Actual yield of ethanol (g/L)	Fermentation Efficiency (%)
Wheat straw	21.7	11.7	11.3	92.3
Rice straw	17.8	9.6	10.6	82.8
Corn stover	16.5	7.8	9.5	91.5
Cotton stalk	18.6	10.8	9.7	90.5

Ethanol production from biomass samples Mean ± standard deviation

(1.5%) and reaction time of 15 minutes. The optimum condition for corn stover at alkaline pretreatment condition was temperature 100 °C, concentration of sodium hydroxide (1.5%) and retention time applied was 20 minutes .

Ethanol recovery

In the conventional process of producing ethanol biofuel from corn starch, the recovery of ethanol from the fermentation broth is accomplished using a multicolumn distillation system

which yields an ethanol-rich stream near the ethanol–water azeotrope of 95 weight % ethanol could be possible depending variety of biomass used

The identification of peak as based on the retention time t_R .Identification of glucose in five samples i.e. wheat straw, rice straw corn stover, cotton stalk and peel wastes were confirmed by the known standard injected through HPLC and its only one prominent peak was observed at a retention time of 3.255 minutes (Table 5).

Table 5. Analysis of sugar by using optimized condition by using HPLC

Components	**Retention time**	**Rice straw**	**Wheat straw**	**Cotton stalk**	**Corn stover**	**Peel wastes**
Glucose	8.6	22.52	28.3	17.5	16.7	12.4
Cellobiose	7.1	1.02	1.05	1.3	1.4	1.5
Xylose	11.6	4.3	5.6	4.7	4.5	3.8
Arabinose	12.0	1.4	1.8	1.4	1.6	1.2
Mannose	13.2	1.5	2.8	2.1	2,5	1.8
Galactose	15.5	1.2	1.5	1.3	1.4	1.1
Furfural	42.5	1.4	2.65	1.3	1.5	1.2
HMF	28	1.2	2.84	1.6	1.7	1.4

Analysis of sugar with HPLC

Protease: Enzyme that hydrolyzes proteins to peptides and/or amino acids. The use of certain proteases in ethanol fermentation has been proven to improve fermentation in the following

ways: • Faster Fermentation Time • Higher Ethanol Yields • Enhanced Yeast Growth • More Efficient Filtration and Evaporation in downstream process steps • More consistent fermentation • More carbohydrate fermented • Reduced carbohydrate in thin stillage

Yeast require certain nutrients to grow and maintain their population in order to convert glucose into ethanol. These may include the following: • Free Amino Nitrogen • Peptides and amino acids • Vitamins and Minerals (Inositols, Zinc, etc.) If yeast nutrition is not maintained, then the fermentation will suffer and result in lower rates and yield of ethanol formation. Nitrogen sources such as Urea, Ammonia, etc. can be added. However, this tends to give only Free Amino Nitrogen.

DISCUSSION

For the production of alcoholic fuels (Butanol and Ethanol) from lignocellulosic feedstock required various technological steps like acid or alkali pretreatment, saccharification and fermentation. To accomplish an cost effective production of biofuels, proper adjusting of all units of system is of great important. In the past different countries significantly improved alcoholic fuels production by refining different process like pretreatment, enzymatic hydrolysis, fermentation, and higher level of ethanol recovery (Yoon *et al.*, 2007). The popular cases of biomass based fuels production in developed countries may be good references for the developing countries . In addition many novel ideas, such as biorefinery and the concept of oriented conversion of classified composition have been investigated for ethanol production. Similar technology are also applicable for butanolproduction fromlignocellulosic biomass (Zhang *et al.*, 2004). The cost of fuels may further decreases when it will produce at industrial scale and efficient combination of these processes will result in competitive biofuel production from plant biomass, which is currently not being utilized effectively.

Fermentation of available sugars in cellulosic biomass have potential to provides important products like acetone, butanol, ethanol and similar other alcohols, that could be used as liquid fuels. Mostly available source of biomass containing carbohydrates are wood wastes , agriculture crops like wheat, rice and cotton straws , corn covers, sorghum straws, fruit and vegetable wastes and similar other substrates. Cellulose is considered as major sugar for alcohol (fuel) production and cellulose is complex sugar present in plants materials. This complex cellulosic material is break down into smaller units with help of acid treatment and enzymatic hydrolysis as well as bacterial/ fungal fermentation. These forms of alcohols is important because that may use as fuels. Therefore biofuels may provide solution of (1) combating climate change, as it help to reduce level of carbon emission release from traffic etc. (2) . Biofuel is able to respond growing demand of fossil fuel and energy (3) Biofuels securing energy supply as it provides security to challenges rising for fuels globally (4).Reducing amount of waste and utilizing natural resources, therefore biofuels is excellent example to provide answer of circular economy . In current study various cellulosic materials was used to produce bioethanol and biobutanol. Therefore various order of alcoholic fuels production from cellulosic substrates was obtained . Among all substrates of biomass used straws has provided better yields of alcoholic fuels as compared to others material used . However, amount of acetone, butanol and ethanol produced depends on nature of cellulosic biomass used as well as various distillation process conducted after fermentation for purification of these type of alcohols.

CONCLUSION

The country ‘s energy demand is expected to increase three fold by 2050, but supply position is not inspiring. Due to similar situation renewable and sustainable energy resources are the best alternative of conventional fuels and energy sources

Bioconversion of lignocellulosicbiomass into alcoholic fuels (butanol and ethanol) provides a sustainable and economical pathway . While, a deep understanding of fundamentals of various pretreatment processes and development of more efficient and economical fermentation processes needs continuing efforts. Moreover, the development of cost-effective detoxification, more efficient microbial strains are required. The process of integration and optimization to reducing energy consumption as well as to increase yields of alcoholic fuels from raw materials could decrease its cost of production and make it more economically competitive

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Volume 1, Issue 2. August, 2021. www.jbbt.org

NILI-RAVI BUFFALO PERFORMANCE IN RESPONSE TO DIETARY NDF

Mini Review

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ABSTRACT

In recent scientific approach, the fiber contents of feed stuffs have been fractioned into acid detergent fiber (ADF) and neutral detergent fiber (NDF). NDF is more complete measure of total fiber since it measures all of the cellulose, hemicellulose and lignin in the diet. Optimum level of NDF contents in ration is important not only in regulating voluntary intake but also may improve the milk production, milk composition, body weight change, dry matter intake and NDF digestibility. A proportional forage increase in the diet decreases voluntary dry matter intake and high forage intake results in lower rumen digestibility. Hence fiber is considered to be the negative index of voluntary intake. In early studies, the dietary NDF less than 25 percent depresses milk fat and lactating buffalo fed diet containing above 28 percent NDF produced

more milk with higher milk fat & protein than those that consumed diets containing 32 percent NDF. But concerning the Nili Ravi buffaloes in Pakistan most of research summarized that 33 % and 38 % NDF contents on dry matter basis in terms of Milk production (4 % FCM) & composition in lactating and weight gain in Nili Ravi buffalo heifers, respectively is optimum. It is concluded that Nili Ravi buffalo is better NDF convertor as compared to cattle and there is provision to conduct research on the other breeds of Buffalo also in Pakistan to prepare NDF based ration.

Keywords: Nili Ravi buffalo, Neutral detergent fiber, Digestibility, voluntary feed intake

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INTRODUCTION

The reduction in roughage content of the ration as a result of high grains is closely related to the change in milk fat and is associated with metabolic problems such as, acidosis, hoof problems, displaced abomasums, liver abcesses and a general decline in health. Adequate fiber and quality promotes good health and better performance. In feeding lactating buffalo there is economic advantage in using a maximum amount of forages and by product feedstuffs. In the newer system of identifying fiber, the fiber content of feedstuffs has been named according to the laboratory procedure namely, acid detergent fiber and neutral detergent fiber. NDF is more complete measure of total fiber since it measures all of the cellulose, lignin and hemicellulose in the diet. Hence optimum level of NDF contents in the diets must be assessed in term of milk production and growth rate to get the optimum performance in Nili Ravi buffaloes. An application of varying level of contents in diets concerning the physiological and production traits is discussed below to understand the phenomenon of the optimum fiber level in terms of NDF contents.

Effect of NDF contents in ration on voluntary intake.

The diets of ruminants must contain a minimum concentration of low energy roughages for proper function of the rumen and signals from ruminal distension to control feed intake when the drive to eat is high. Basically the effect of diet on feed intake varies with the physiological stages of animal. The objective of this review paper is to determine the effect of NDF contents of ration on voluntary feed intake in Nili Ravi Buffaloes. Basically the information about intake is very important because a cow/buffalo require quantities rather than percentages of nutrients. Fiber is considered to be the negative index of feed intake. Van Soest, (1994) in their respective studies concluded that feed intake can be limited by the bulkiness (fill effect) of the feed in relation to the voluntary intake of the reticulo-rumen. This is the characteristic of diet that describes the physical regulation of intake. Further, it is explained that ruminant's reticulo-rumen volume determines the potential physical intake of forages. The bulkiness of diet is related with the fiber part. Earlier studies showed that forage NDF in diets can inhibit feed intake. A proportional increase in forage in diet decreases voluntary dry matter intake (Dado and Allen, 1995) and diets with high forage contents usually exhibits lower rumen digestibility. Obviously such diet spends longer time in the rumen, pass through slowly, create more distension and thus reduce intake.

Distension in the rumen is determined by both the weight and volume of the digesta. Percentage of neutral detergent fiber of the diet in the voluntary intake of animals plays a crucial role. In this respect, Dado and Allen (1995) demonstrated that in early lactating cows 35 % NDF diets restricts DMI but DMI was not limited when 25 % NDF diets were fed with or without inert bulk in the rumen. Allen, (2000) summarized fifteen studies and concluded that there was a general decline in voluntary dry matter intake with increasing NDF concentration in the diets above 25% NDF. Likewise a research conducted by Adin *et al.* (2009) fed two diets containing 12.8% (experimental) and 18.7% (controlled) roughage NDF. According to the results there were 7.2% higher dry matter intake experimental cows than in controlled cows. Although most of the researchers has reported a significant decrease in dry matter intake as forage neutral detergent fiber increased, the dry matter intake response was variable, that was depending upon the degree to which intake was limited by fill of the rumen. High producing cows are limited by fill to the greater extent and the filling effect of forage fiber varies depending upon characteristics of fermentation and particle size. Similarly it has also reported that NDF and ADF decreased as ration energy content was increased. However maximum intake of digestible energy was observed when the level of neutral detergent fiber was 40 to 44% and level of acid detergent was 16% in the diet of cow. This study showed that if dietary fiber content was beyond the optimum level, then reduced the animal performance. Dry matter intake have been limited when cows producing approximately 40kg of milk/ day were fed feed with more than 32% neutral detergent fiber. Kendall *et al.* (2009) showed that dry matter intake was greater for cows consuming diets with 28% NDF. Likewise other scientists reported that the intake is limited by the capacity of animal to consume dietary NDF, estimated as 1.2% body weight of a ruminant animal.

Effect of NDF contents on milk production and composition.

Proper NDF ratio in ration is important to get optimum milk yield from the animals. In a study conducted by Dado and Allen, (1995) showed that cows produced 5.2 kg/day more milk and consumed 5.1 kg/day more dry matter when fed low fiber diets (25 % NDF) compared with high fiber diet (35 % NDF of dietary dry matter) on iso-nitrogeneous rations. In dairy cattle, increased

dietary NDF concentration would likely increase milk fat which might partially compensate, from an economic standpoint, for lower milk production associated with increased net energy intake. Adin *et al.* (2009) conducted an experiment on total mixed ration fed to dairy cattle containing 20.5% less NDF in experimental ration than control total mixed ration (11.7 vs. 14.1% of DM respectively). By this way a favorable condition for NDF digestion was created in the rumen of experimental cows. The advantage of the experimental cows in intake and digestibility were reflected in associated increase of 7.4% in milk production and of 9.2% in FCM yield as compared with control cows. Yang and Beauchemin, (2005) prepared 3 diets consisting of high (11.5%), medium (10.3%) and low. 8.9%) NDF contents and observed significant effect due to dietary NDF to a greater extent than other nutrients. However increased digestibility due to increased dietary NDF did not significantly improve milk production or milk composition. Another study conducted by many scientists fed diets to early-lactation cows that were formulated to be low or high in fiber fill value and that had been formulated to differ in rate and extent of NDF digestion, although cows produced significantly more milk and milk protein on the low fill diet. Likewise another research conducted by Greter *et al.* (2008) on dairy heifers. They prepared 3 diets, control diet containing silage, diet with 10% straw, diet with 20% straw. According to the results there was a linear decrease in dry matter intake of the heifers with the addition of straw to the diet as well as there was a linear decrease in consumption of crude protein, ADF, NDF, non fibrous carbohydrates, and total digestible nutrients.

Diets having with 25% NDF resulted in similar milk production with a similar composition of milk as did diets with higher NDF concentration. In these studies dietary dry matter contained 16 to 20% NDF from forages. Many studies depicted that diets with less than 25% total NDF and less than 16% NDF from forage depressed milk fat percentage. Kendall *et al.* (2009) reported that cows fed diet consisting of 28% NDF produced more milk, fat, and protein than those cows who consumed diets containing 32% NDF. According to NRC, (1989) a minimum amount of 28% NDF is needed in dairy cattle diet, but it is reduced to 25% during the time of high milk production in order to maintain normal milk yield and milk fat and to minimize digestive disorders. Likewise Hoffman and Bauman (2003) conducted an experiment on dairy cows. They used total mixed rations with different consistency of NDFD contents such as 45.0%, 50.0%, and 55%. According to the results there was more dry matter intake and more milk production when fed forages that had a higher NDF digestibility. Ivan *et al.* (2005) reported that milk yield per ton

of corn silage on dry matter basis was 168 lb. higher for high fiber than low fiber corn silage by feeding high fiber (53% NDF) and low fiber (49% NDF) corn silage in 30% NDF diets respectively. But the research conducted by Knight *et al.* (2005) fed lactating dairy cow alfalfa hay that contained either low neutral detergent fiber 36 to 37% of diet dry matter or high neutral detergent fiber such as 41 to 42% of dry matter. Results have shown that milk yield was not increased by the higher neutral detergent fiber digestible alfalfa hay. Likewise the research conducted by Mertens (1994) fond a relationship between 4% fat corrected milk yield and neutral detergent fiber content of the ration and concluded that there was a maximum milk production when cow was fed ration with 35% NDF contents on dry matter basis. Likewise another research conducted by Holt *et al*.(2010) on dairy cows, they conducted an experiment to determine the effect of corn silage and non forage fiber sources in high forage diets which was formulated with alfalfa hay and corn silage on production performance in lactating dairy cows. Production of milk protein is economically important to dairy producers and milk manufacturers. When we feed forage that is higher in NDF in the diet that may increase the milk protein contents as described by Hoffman and Esser (1997).

Effect of NDF contents on body weight change

The research conducted by Adin *et al.* (2009) who prepared two total mixed rations of varying level of NDF. Experimental TMR contained 20.5% less physically effective NDF than control TMR (11.7 verses 14.1% of dry matter respectively), There was non significant difference with respect to efficiency of utilization of feed on milk production and body weight gain. Likewise Ware and Zinn, (2004) conducted a trial on Holstein steers and used fiber in a range (4-8%), which did not limit rumen function and growth performance. Lippek *et al.* (2000) conducted an experiment on steers to determine the effect of supplementry fiber and grain on weight gain. They concluded that there was no change in body weight gain at different supplementary fiber levels in total mixed rations.

CONCLUSION

In early studies, the dietary NDF less than 25 percent depresses milk fat and lactating buffalo fed diet containing above 28 percent NDF produced more milk with higher milk fat and protein than

those that consumed diets containing 32 percent NDF. But concerning the Nili Ravi buffaloes in Pakistan most of research summarized that 33 % NDF contents on dry matter basis in terms of Milk production (4 % FCM) and composition and 38% in growing Nili Ravi Buffalo Heifers for growth parameters is optimum. It is concluded that Nili Ravi buffalo is better NDF convertor as compared to cattle and there is provision to research on the other breeds of Buffalo in Pakistan to determine the optimum level of NDF in their diets and it is suggested that NDF content is 2 to 3 times as important as fiber digestibility in affecting production and intake. Thus, rations should be formulated first to obtain proper NDF content in buffalo breeds and then NDF digestibility can be used to fine -tune rations.

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Volume 1, Issue 2. August, 2021. www.jbbt.org

SCREENING AND OPTIMIZATION OF SUBMERGED FERMENTATION*OF ASPERGILLUS* SPECIES FOR *KOJIC ACID*PRODUCTION

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ABSTRACT

Kojic acid is organic acid obtained from numerous species of *Aspergillus* through fermentation.This is among most demanding substances in cosmetic industries as an alternate to carcinogenic Hydroquinone and has grabbed a vital position in Pharmaceuticals, Food and Agriculture industries. Current experimental approach was designed for production and purification of *Kojic acid* crystals from *A. flavus*and *A.oryzae* and measured the effects of pH, temperature, static and non-static (shaker) condition on *Kojic acid* yield in submergedfermentation. Significant yield of *Kojic acid* crystal was obtained by *A. flavus*as compared to *A. oryzae*. Optimized conditions were pH 4.5 for (*A. flavus*) and 3.5 for (*A. oryzae*) at 30 ^{0}Cwith 20 days of incubation. High yield of *Kojic acid* crystals were produced under static condition (16 g/L in *A. flavus*and 11 g/L in *A.oryzae*) in contrast to non-static (shaker) conditions (6 g/L in *A. flavus*and 5 g/L in *A. oryzae).* Quantitative estimation of *Kojic*

acid was done through Bentley's colorimetric method followed by TLC, FTIR and HPLC. This analysis was found successful after achieving the high yield of *Kojic acid* under optimized conditions.

Keywords:*Kojic acid, A. flavusand A. oryzae, pH, Quantitative, TLC, FTIR, HPLC*

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INTRODUCTION

Kojic acid (5-hydroxy-2-hydroxymethylgamma-pyrone; KA) is considered as amajorsecondarymetabolitewhich is produced by the carbohydrates through various microorganisms, such as *A.oryzae*, *A.flavus,* and *A. tamarii*, as well as *Penicillium*species andcertain bacteria (Hazzaa *et al*., 2013; Antonius *et al*. ,2019).*Kojic acid* is an organic acid which covers almost all the fields, act as antibacterial, antifungal, anti-melanosis, and a chelating agent. It can be applied in various areas of science such as chemistry, health, food, and cosmetics; however, its current, reported uses are limited at commercial scale, as its despites at the market level were being developed about 40 years ago. However it is also marked as an organic chelation Agent used as a decolorizing agent. It is wellknown for its uses in Cosmetics Industry. *Kojic acid* is reported to have an antioxidant potential due to which is marked as to be used in the cosmetics industry, which has been further described as an alternative to hydroquinone for the lightening of the skin (Kady *et al*., 2014). This acid was basically discovered from the mycelia of *A. oryzae* which was grown on steamed rice (the term koji means steamed rice in Japanese) in the year 1907 (Burnett *et al*., 2010). Koji stands for rice in Japanese language and likewise the name of *Kojic acid* was given to this acid (Brtko *et al*., 2004).

Kojic acid can be produced in large amounts by using various carbon and nitrogen base sources, based on agriculture waste under aerobic fermentation process. Till now among others glucose has been marked as the one of the best source as a raw material in order to attain higher yields of *Kojic acid.*Therefore use of some mutated strains of *A.flavus*had shown remarkable peaks f *Kojic acid* used potato,sago and corn starch etc. (Chaudhary *et al*., 2014). *Kojic acid* has many economic applications in different eras, such as in the field of medical, it has been reported as having anti-bacterial and anti-fungal potentials. While in case of chemical industries it has been also used auspiciouslyfor the synthesis of azo-dyes andbiodegradable compounds. Furthermore in the field of foodindustries, it has been used as an anti-speckand anti- melanosis (blacking of product) agent for agricultural products (Hassan *et al*., 2014).

On commercial scale, *Kojic acid* synthesis was initiated by Charless Pfizer and Company of USA in 1955 which was the first company for this production. This finding was useful for further enhancement at commercial value, applications and production (Brtko *et al*., 2004; Bentley, 2006). Recently the production of KA is marked by two Chinese and three companies in Japan, Switzerland, and the USA.

Rapid growth of industries and discovery of the potential applications of KA along with its derivatives, generated a demands for the manufacture of this product KA greatly (Saeedi *et al*. ,2019).

According to global marketing report the use and production of cosmetic is increasing day by day. KA is used as primary constituent in skin products as it obstruct the synthesis of pigment, responsible for skin blackening (Masse et al. 2001). It is a good for the prevention of melanin production which ultimately safe man from freckles. However it was banned in Asia, later on due to carcinogenicity in by Drug Authority (Emami *et al*, 2007).

Kojic acid is present in colorless prismatic needle form that can sublime in vacuum with no alteration in morphology. It has melting point ranging from 151^{o}C to 154^{o}C, while the boiling point of this substance is 401.67^{o}C at 760 mmHg. Furthermore the extensive use of this acid in the production of cosmetic are due to the ability of acting as a UV protector, which extensively work as a suppressor of hyperpigmentation in human and could have great potential to restrainer the formation of melanin, which is basically associated with its *tyrosinase* inhibition. This could also be developed as a chemo sensitizer in order to increase the potential of antifungal drugs or fungicides at market levels (Saeediet al. 2019).

According to Cryoscopy Method, molecular weight of *Kojic acid* is 142.1 and its maximum peak of UV Absorption Spectra is at 260-284 nanometer. KA is marked assoluble in Water, Acetone, Ethyl Acetate, and Ethyl Ether & Ethanol and also slightly soluble in Ether, Alcohol-Ether mixture, Chloroform & Pyridine etc. The solubility of this acid in Pyrimidine is very low, however, it is completely insoluble in Benzene (Neil, 2006).

Kojic acid has properties of weak acids and it is categorized as a multifunctional, reactive Gamma-Pyrone. Its reactivity predominantly could be observed at all positions on a ring and a number of valuable industrial products can be made from it such as metal chelates, Ether, Pyrimidine, Pyridones, Azodies, Mannich Base etc. This is also reported as a strong antibacterial component, and had shown very strong control against gram positive bacteria (Zohri *et al*., 2018). Since beginning, several numbers of its chemical reactions have been studied for research purpose and its deliberate use for the benefit of humankind. It has ability to form salt with rare metal such as sodium, zinc, cooper, calcium, nickel and cadmium as its Hydroxyl group acts as weak acid at carbon 5 position. Carbon 5 side chain acts as primary alcohol whose reactivity is increased by neighboring oxygen atom in the nucleus (Chaudhary *et al.*, 2014).

It has strong reactions with amino acids to form conjugates, which exhibit a higher Tyrosine inhibition activity and stability as compared to KA alone, its derivatives are also reactive. (Mohammad *et al*., 2010; Noh *et al*., 2009). These derivatives are efficiently utilized in human medicines, veterinary medicines and synthesis of new and high biologically active compounds with preferable properties (Brtko, 2004). Secondary metabolites are the organic compounds which are not directly involved in the procedure of normal growth, progression and reproduction of an organism. Primary metabolites are responsible for the production of Secondary metabolite. Secondary metabolites play vital role in an organism's growth, reproduction as well as defense mechanism of living organism.

Plants use secondary metabolites in their defense system against herbs and humans utilize secondary metabolite as medicine, recreational drugs, texture preservative, food thickening and flavoring agent. (Pichersky and Gang, 2000; Gök *et al*., 2013). Natural selection has been very important in the discovery of vital compounds which can give rise to new products. Organisms that inhabit vital biotopes produce natural products that perform specific functions and they can be used for vital secondary metabolite. (Schulz *et al*., 2002). However certain plants of *Solonaceae*had been reported to be used in drugs to cure diahorea and vomiting, phytoestrogens is secondary metabolite found in seed of Neem plant and act against insects (Chizzali and Beerhues, 2012).

Aspergillus is one of the oldest known fungus known for the production of metabolites, acids and enzymes. Industrial utilization of microbial enzymes started 100 years ago in western world with the

synthesis of Alpha Amylase by *A. oryzae*. These enzymes are used for numerous applications like cheese production, starch production, clarification of juice, food preservation and instant tea making. Thought the strains of *oryzae*had been also reported for the production of lipase (Ahmed et al. 2019).*Aspergillus* is widely used for the production of organic acids such as citric acid, gluconic acid, KA at industrial level. These organic acids are used in food, soap, cream and in pharmaceutical as well. In course of survey different fungal species were used for its synthesis but *Aspergillus* species show fairly high yield of KA. They give deep red color with ferric chloride and upon continuous extraction produce crystals of KA (Brian, 1951).

According to 2014 Global marketing report, cosmetics business was 460 billion US dollar in 2014 and it is expected to reach to 675 billion US dollar in 2020. Hydroquinone used in skin care product has been replaced by *Kojic acid* (Emami *et al.*, 2007). Currently, *Kojic acid* is frequently used as a basic ingredient in skin lightening products because of its ability to block the formation of melanin, a pigment responsible for dark skin. (Masse *et al.* ,2001).

The main objective of this experiment was to investigate the highest potential strains for the production of KA among *A.flavus*and *A. oryzae*isolates through submergefermentations techniques. For that fermentation procedure was optimized by varying condition as static and non-static, temperature and pH of the media was also investigated.

MATERIALS AND METHODS

Experimental Lay Out

Experiment was arranged by providing varying pH and Temperature in Static and Non-static conditions to attain Optimized conditions of fermentation Procedures for the highest yield of Kojic acid.

Collection and Maintainance of Fungus

In order to carried out this experimental analysis two species of *Aspergillus* (*A. flavus*and *A. oryzae)* were purchased from First Culture Bank of Pakistan, University of the Punjab Lahore. For the enhancement of the growth of mother culture, Sub-cultured by using potato dextrose agar (PDA) as the best source for the production of Micro-Organisms. Formation of Potato Dextrose Agar (PDA) media 3.9 g of PDA was dissolved in 100 ml of water in a 250 ml of Flask and were placed on hot plate for mixing until thoroughly dissolved in water. Subsequently, the flask containing the solution were cotton plugged and wrapped by aluminum foil. The flask was autoclaved for 1h, then poured into tubes and placed for ten min. After solidification of PDA solution, a loop of mother culture

was taken and streaked on the Slants. These Slants were covered tightly and placed into incubator for 7 days at 30^{o}C. The whole procedure of Slant formation was conducted in a Laminar Flow to avoid contamination hence enhancing the number or growth of mother culture (Syarifuddin and Saidi, 2013).

Preparation of Culture Media

One liter of culture media was prepared by using 100 g ofglucose (as a carbon source), 5 gyeast (as a nitrogen source), 1 g KH_2PO_4, 0.5 g $MgSO_4$and 10 ml methanol. Aforementioned media being the high productive media was utilized by all referred Scientists (El-Aziz, 2013). To avoid the production of Aflatoxins, Methanol was used in the media solution (Madihah et al. 1998).

Maintenance of pH and Temperature

150 ml of Culture media was poured in 250 ml flask and twelve such numbers of flasks were prepared. pH of these flasks were adjusted to 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 by using H_2SO_4 and NaOH. 2

such flasks were prepared for each pH, 01 for Static and the other for Non-static (shaking) procedure. All flasks were labeled with (pH and target procedure)-wise and inoculated with *A. flavus*and *A. oryzae* separately. Then 06 flasks containing the solution ranging from 3.5 to 8.5pH were placed on shaker at 30 ^{0}C and likewise 6 others in an incubator at 30 ^{0}C for 20 days.

150 ml of Culture media was poured in 8 prepared 250 ml flasks varying temperatures under 2 different procedures i.e. Static and Non-static (shaking). All flasks were labeled with temperature of 25, 30, 35 and 40 ^{0}C and target procedure wise and inoculated with *A. flavus*and *A. oryzae* separately in static and non-static conditions for about 20 days.

Wet and Dry Mycelia Weight

The flasks were removed from shaker and incubators and Mycelia mass was separated from culture and weighed (Weighing balance Model No. JJ224BC). The same mycelial mass was placed in oven to dry for 24 h and weighed again.

Analysis of Kojic acid (KA)

Colorimetric method was used for the quantification of *Kojic acid* (Bentlay, 1957). 1 ml of diluted sample was mixed into 1 ml $FeCl_3$ solution (1g of $FeCl_3.6H_2O$ in 100 ml of 0.1N HCL) (Sikem, 2013). Sample gave a reddish purple color due to reaction between hydroxyl and phenolic group as KA makes a complex with $FeCl_3$ resultantly giving this purple color. Absorbance of the sample was measured at 500 nm by using UV- VIS Dual Split-Beam Spectrophotometer (Model No. UV2400PC) and equivalence was checked by *Kojic acid* standard curve (Crueger and Bentley, 2006).

Comparison of Kojic acid Concentration By Standard Curve

Comparison of Standard Curve of self-prepared *KA*and prepared from standard *KA*obtained from PCSIR, Lahore was drafted to evaluate the comparative yield.10 different dilute solutions of Standard *Kojic acid* were prepared and placed in Spectrophotometer (Model No. UV2400PC) at 500 nm to measure absorbance of dilute solutions and plotted a graph between the concentrations of multiple dilute solutions and absorbance (Figure 1). Later, need-based dilute solutions of self-prepared *Kojic acid* (culture media) were prepared and likewise placed in Spectrophotometer (Model No. UV2400PC) at 500 nmto measure absorbance of dilute solutions. Trend analysis w.r.t. absorbance of dilute solutions was noted after intervals of 5 days and obtained O.D. of Standard and self-prepared *Kojic acid* were compared to evaluate the comparative yield for both.

Extraction and crystallization of Kojic acid

Extraction of *Kojic acid* crystals was carried out by filtration of culture media by Watt man filter paper No. 1. The left mycelial mass was further used for wet and dry weight procedure as mentioned above. The left over solution was treated with ethyl Acetate and placed at 5^{0}C in refrigerator (Model No. FYLC-400/FYLC-600)for 24 h. Crystals of KA were seen next day at the bottom of beaker, than filtration was carried out to attain them which were oven dried at 80 ^{0}C for 01 hour (Figure 2 A and B) (Chaves, 2012).

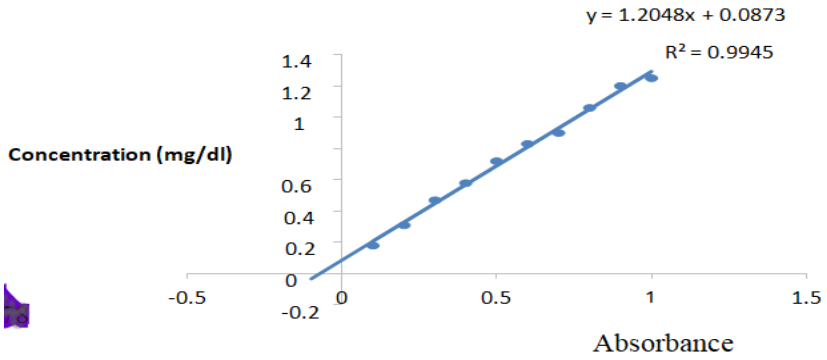


Figure 1 Standard Curve of Pure *Kojic acid*

Figure 2 A: extraction of *Kojic acid*; B:*Kojic acid* Crystals **C:** *Kojic acid* on TLC plate.

Thin Layer Chromatography (TLC)

It was carried out to authenticate the presence of *Kojic acid* in culture media. Three Dilute solutions; One for Standard *Kojic acid* and two for *A. flavus*and *A. oryzae* (self-prepared *Kojic acid*s) were prepared (1 ml water should contained 1 mg of *Kojic acid*)in each dilute solution to further perform TLC procedure by using TLC plates coated with silica gel (Kisel gel160F 254) and a line marked with A (standard *Kojic acid*), B (*Kojic acid* prepared with *A. flavus)* and C (*Kojic acid* prepared with *A. oryzae*) (Fig 3 A). All 03 solutions were dropped on the plate and placed in mixture of water, acetic acid, acetone and ethyl acetate (1:1:3:5). After 15 minutes the plate was taken out and placed until dried. A solution of $FeCl_3$(1 g $FeCl_3$ with 850 µl of HCl and 100 ml of water) was sprayed on TLC plate to obtain observable spots of poured *Kojic acid* (Figure 2 C).

Fourier Transform Infrared Spectroscopy (FTIR)

To further authenticate the presence of *Kojic acid* in extracted crystals, Fourier Transform Infrared Spectroscopy (FTIR)at FTIR spectrometer (a Shimadzu Prestige 2, Apodization: Happ-Genzel spectrometer) had been performed by ACRC Department of PCSIR laboratories, Lahore, Punjab, Pakistan. Each spectrum was recorded from 4000 to 500 cm^{-1} and 64 scans were accumulated for each spectrum.

High Performance Liquid Chromatography (HPLC)

It has been done as described by the protocol of Asma et al. (2018). Crystals of *Kojic acid* has been dissolved in 80 mL C_2H_5OH (80%), for one minute, filtered and stored at minus 20 ^oC till analysis. 5 mL aliquot of stored extract was dried under vacuum at room temperature and resuspended in one mL C_2H_5OH (80 %) and filtered through 0.45 um nylon 66 filter paper and injected 10 µL from this solution into the HPLC system. Perkin-Elmer model of HPLC with binary LC pump of 250, an LC 600 auto sampler, a UV/V with spectrometric UV detector (at 270 nm)of LC- 290, PV Nelson 900 series INTERFACE, Hewlett- HewlettPakkrad 3394 Integration and a Bondapak C-18 column (250*4.6 mm). Mobile phase was 0.5% orthophosphoric acid in CH3 OH; 1 mL/ minute flow rate was set and Aglycone content was measured using Quercetin dihydrate (95%) as standard. Concentration was measured by using following formula

$$\text{Concentration of } \textit{Kojic acid} = \frac{\text{Peak Area of Sample}}{\text{Peak Area of Standard}} \times \text{Concentration of Sample}$$

Patil et al. [13]. Twenty grams of onion sample, by removing dry leaves, was grounded with

RESULTS AND DISCUSSION

Effect of pH on *A.flavus*:

In the past, a number of research analysis on synthesis of *Kojic acid* lead to the conclusion that numerous *Aspergillus* species have ability to produce *Kojic acid*. During this work the impacts of varying pH was closely examined on the production of *Kojic acid* in submerge fermentation. It was found that at 20^{th} day the maximum yield of *Kojic acid* crystals as g/L was obtained (13, 16, 9, 7, 5 and 4 under static culture condition and 8, 10, 6, 4 and 3) under non-static at pH 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 correspondingly. Moreover maximum amount of *Kojic acid* crystals formulated from *A. flavus*was obtained at pH 4.5 under Static and Non-static culture media.The highest yield of *Kojic acid*crystals at pH 4.5 under static and Non-static conditions was 16 g/L and 10 g/Lrespectively. Increase in pH of the culture media resulted negative impacts on quantitative concentration of *Kojic acid* and pH 4.5 was found to be most appropriate for the highest yield of *Kojic acid*. On the other hand, culture media of *A. flavus*at Non-static conditions gave low yield as compared to the ones treated at Static Conditions (Figure 3).

The subject research study results were found to be parallel to the results ofHazza *et al.*, (2013). Effect of high pH was significant on fungus metabolism. The metabolism of fungus shunted to some other pathways instead of *Kojic acid* production, so yield of *Kojic acid* reduced very much with high pH (Rosfarizan et al.,2010). *A. flavus*showed highest yield of *Kojic acid*crystals at pH 4.0. With an increase in pH of the culture media the growth of fungus and yield of *Kojic acid* ceased (Kady *et al*., 2014).

Effect of pH on *A.oryzae*

Yield of *Kojic acid* crystals significantly affected when high pH and conditions applied. At day 20, concentration of *Kojic acid* was 6, 4, 3, 2, 1 and 0.9 g/Lat pH 3.5-8.5 respectively under Static conditions, whereas, under Non-static conditions the yield was 5, 3, 2, 1.6, 1.1 and 0.5 g/L at pH 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 respectively (Figure 5).

At 20th day, the best yield of crystals of *Kojic acid* has been obtained from *A.oryzae* at pH 3.5 (6 g/L under static and 5 g/L under non-staticconditions). Moreover, Static condition has shown good results of yield as compared to that of Non-static. Acquired results are similar to the results of Hazza, (2013) and Hassan et al., (2014). Same results was explained by Kadyet al., (2014), *A.oryzae*and its strains with an increase in pH give low yield of acid and growth of fungus. *A.oryzae*show high growth on low pH i.e. 2 while high *Kojic acid* production is obtained at pH 3.5. As explained by Basappaet al., (1970), yield of *Kojic acid* severely reduced with a small deviation in pH and *A.oryzae* showed highest yield at pH 3.0 and increased pH decreased both yield of acid and fungal growth.

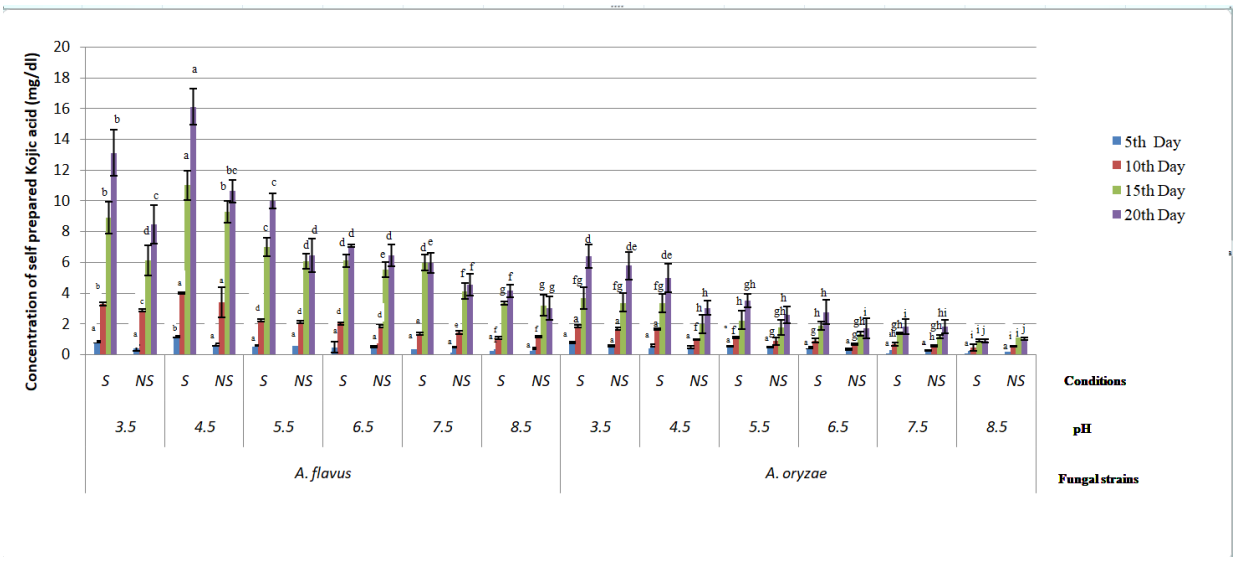


Figure 3 Concentration of self-prepared Kojic acid from *A.flavus* and *A. oryzae* in static and in non-static conditions on different days at different pH

S= static, NS= non-static, a= Maximum amount of *Kojic acid* (as per statistical analysis), j= Least amount of *Kojic acid* (as per statistical analysis)

Effect of temperature on *A.flavus*

Yield of *Kojic acid* crystalswas strangely different at different temperature(s) (Figure 4) after 5, 10, 15 and 20 days. The growth of fungus was good at 25^{0}C to 30^{0}C and extraordinarily high at 30^{0}C and unusually low at 40^{0}C. At day 20 the obtained yield of *Kojic acid* crystals was 6, 12, 5 and 3 g/L at 25 ^{0}C, 30^{0}C, 35^{0}C and 40^{0}C respectively. Highest yield of *Kojic acid* was 12 g/L at 30^{0}C (Gqalen *et al.*, 1997 ; Ito, 2001).

Effect of temperature on *A. oryzae*

Temperature adversely affects the yield of *Kojic acid* as well growth of *A. oryzae*. High growth of fungus was seen when temperature is 25 ^{0}C to 30 ^{0}C (Figure 6). At 30 ^{0}C the yield of *Kojic acid* was amazingly high and at 40 ^{0}C it was extremely low. The yield obtained at day 20 was 2, 4, 1.0 and 0.2 g/L at 25 ^{0}C, 30^{0}C, 35^{0}C and 40^{0}C correspondingly. Highest obtained yield was 4 g/L at 30^{0}C and lowest as 0.2 g/L at 40 ^{0}C. A decrease in *Kojic acid* concentration with increase in temperature was observed, whereas, 28^{0}C was found to be the best temperature by El-Aasar (2006) in *A. oryzae*. Same results are reported by Devi *et al.* (2014) and Rosfarizan *et al.* (2010) that an increase in temperature shows significant effect on yield of *Kojic acid*.

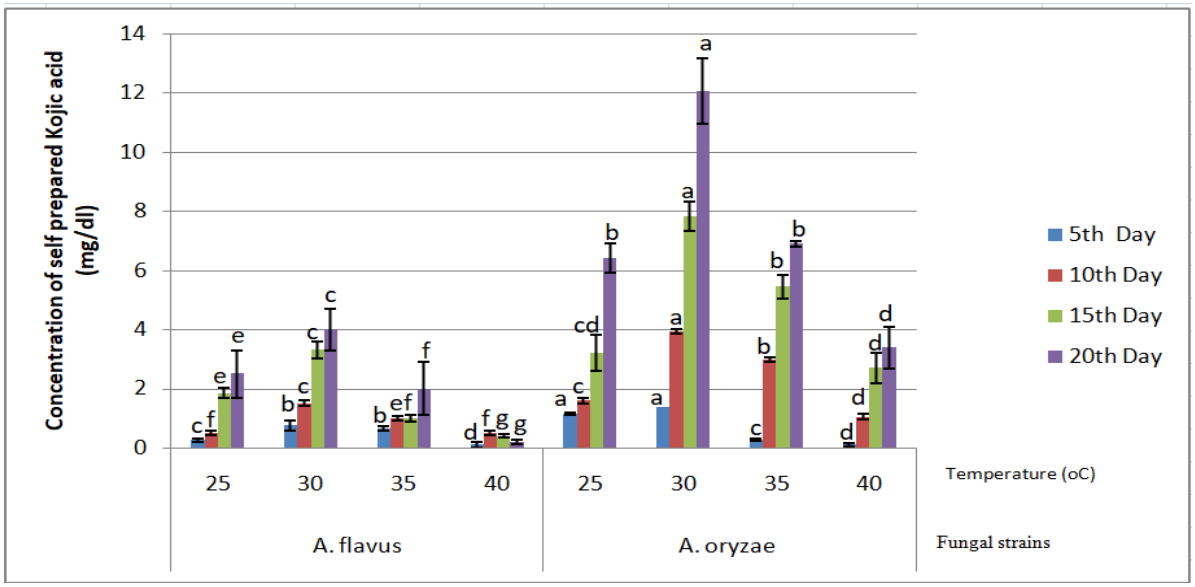
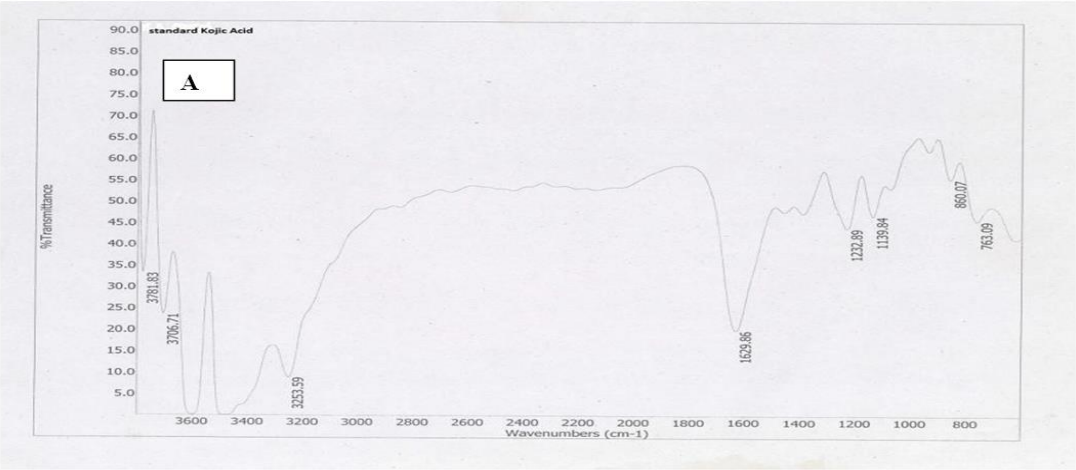


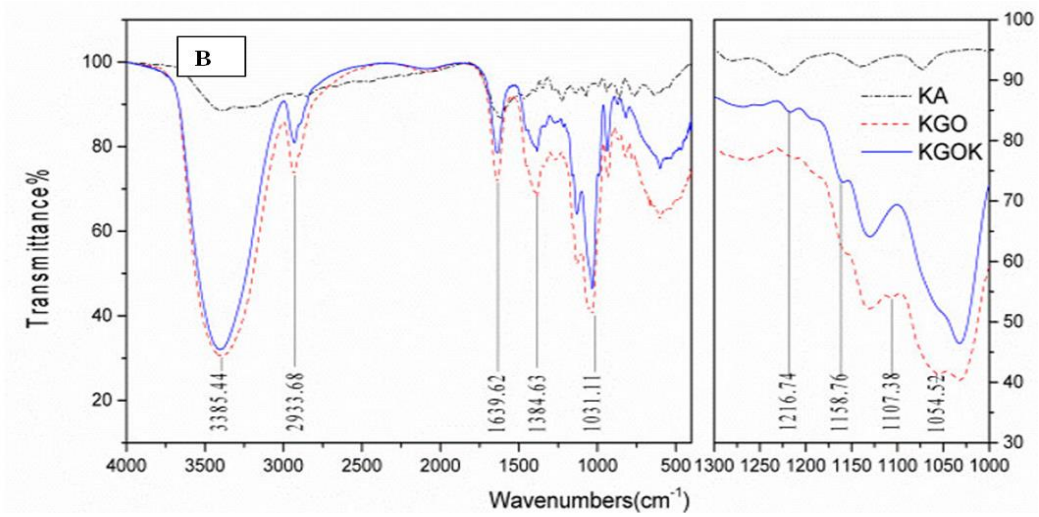
Figure 4 Concentration of self-prepared *Kojic acid* from *A. flavus* and *A. oryzae* at different temperature on different days.

a= Maximum amount of *Kojic acid* (as per statistical analysis), j= Least amount of *Kojic acid* (as per statistical analysis)

FTIR analysis of*Kojic acid:*

FTIR Spectrum analysis of the sample showed the presence of various active functional groups, which had been indicated by the peaks which presents the presence of Amines N-H stretch (at 3253.59 cm^{-1} and 3179.41 cm^{-1})of Crystals. Samples also showed the presence of alkenes at 1629.86 cm-1 (Cyclic-C=C). Furthermore these samples showed the presence ofalcoholic compound (O-H stretch) at 3600 wavelength cm^{-1}(Figure 5).


standard Kojic Acid
A
%Transmittance
Wavenumbers (cm-1)
3761.83
3706.71
3253.59
1629.86
1232.89
1139.84
860.07
763.09


B
Transmittance%
Wavenumbers(cm-1)
KA
KGO
KGOK
3385.44
2933.68
1639.62
1384.63
1031.11
1216.74
1158.76
1107.38
1054.52

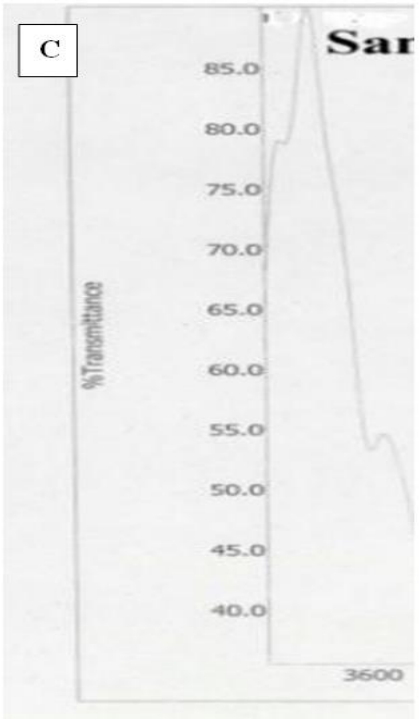
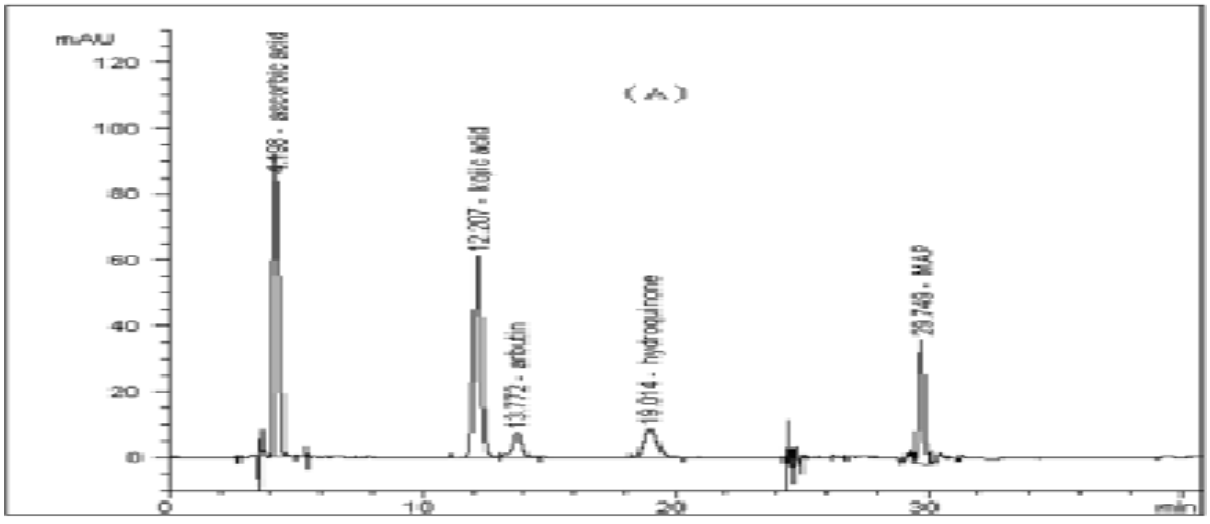


Figure 5 FTIR Spectrum of *Kojic acid* (KA) in the wavelength region 4000-400 cm $^{-1}$ from (A) *A. flavus* (B) *A. oryzae* (C)Standard *Kojic acid* crystals

4.6 HPLC of *Kojic acid:*

The best results obtained by HPLC of*A. flavus*and *oryzae* contained the compound with the same retention time as that of *Kojic acid* standard (Figure 6). All the experimental analysis(HPLC, TLC and FTIR) indicated that *Kojic acid* was produced by both fungus.



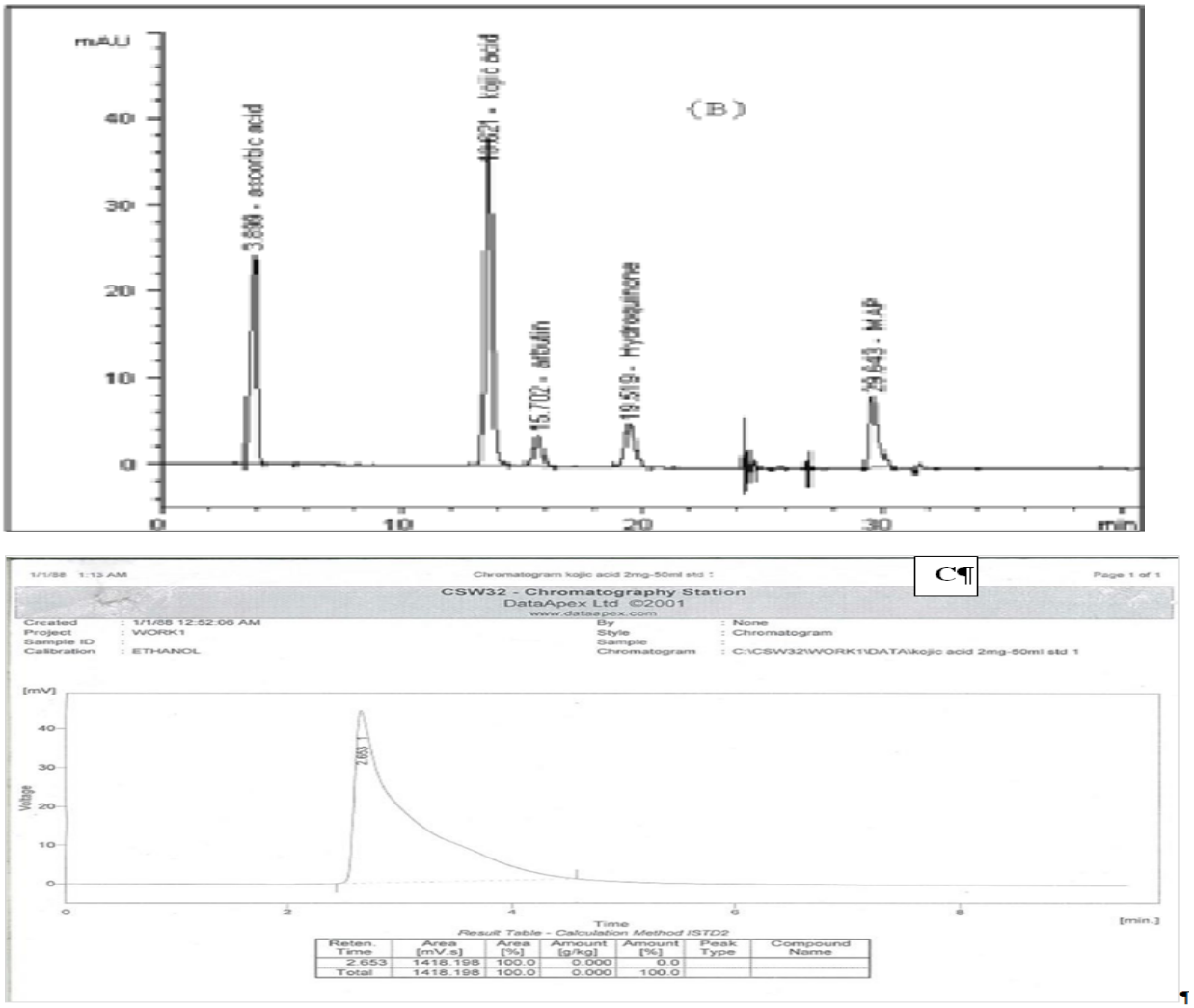


Figure 6 HPLC of standard *Kojic acid* shows *Kojic acid* peak from (A) *A. flavus* (B) *A. oryzae* (C) Standard

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