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Guide rNA





Epidemiology, Pathogenesis and Management of Preeclampsia

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ABSTRACT

Preeclampsia (PE) is a pregnancy associated complication mainly demonstrated by the onset hypertension, proteinuria, foetal growth restriction, and maternal organ dysfunction including renal, cerebral and hepatic diseases before or after 34 weeks of gestation. As a cause of prenatal and maternal morbidity and mortality, it remains a major health concern to females worldwide. In this context, we aimed to provide an accumulative account of recent developments about the risk factors, pathophysiology, consequences and management of PE. Several genetic, acquired and environmental risk factors have been associated with the progression of PE. Pre-existing hypertension, chronic renal disease, family history, obesity, multi-foetal pregnancies and history of miscarriage are main risk factors. The disease is initiated by abnormal placentation, incomplete remodelling of spiral artery, utero-placental hypoperfusion leading to placental hypoxia and ischemia. Many molecules of foetal origin are released in to circulation and result in the dysfunction of maternal endothelium which is the hallmark of PE. The disease also has postpartum impacts on maternal and foetal health, the main consequences include cardiovascular complications, hypertension, diabetes, neurodevelopmental issues and dementia to the mother and offsprings. In the recent past, the revelation of antiangiogenic molecules and pathways has provided critical information about the pathogenesis of PE. Several molecules such as sFLT1 (soluble fms-like tyrosine kinase 1), Eng (soluble endoglin), angiotensin II, PIGF (placental growth factor), and VEGF (vascular endothelial growth factor) are regulated differently during the pathogenesis of PE. These molecules not only offer the candidate biomarkers for early disease detection but also provide therapeutic targets. Many surveillance and management strategies reported in the recent literature have also been included in the present review. Studies have emphasized that PE has a long-term threat to the health of mother and child. Therefore, the development of interventional strategies for the prevention and management of postpartum cardiovascular complications poses a challenge.

KEY WORDS: Preeclampsia, Hypertension, Pathogenesis, Risk factors, Treatment

INTRODUCTION

Pregnancy-induced hypertension (PIH) is characterized as systolic and diastolic blood pressures after 20 weeks of gestation that includes gestational hypertension (GH) and preeclampsia (PE)^{1,2}. According to estimates, more than 10% of pregnant women are affected by hypertension, it may be pre-existing chronic hypertension or pregnancy induced

gestational hypertension. The chronic and gestational hypertension may be differentiated on the basis of their timings i.e. the chronic hypertension is a systolic and diastolic blood pressure above 140 mmHg and 90 mmHg before pregnancy or before 20 weeks and persists after pregnancy, GH is the hypertension that starts due to physiological changes after 20 weeks of pregnancy³⁻⁵. In some cases, hypertension is accompanied by increased levels of proteins in the urine

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known as Preeclampsia (PE). According to the definition by the International Society for the Study of Hypertension in Pregnancy (ISSHP), preeclampsia a condition characterized by a combination of gestational hypertension with organ damage and proteinuria at 20 weeks of gestation⁶⁻⁸.

Preeclampsia affects up to 5% pregnancies worldwide^{9,10} resulting in the death of 0.5 million infants and 76,000 women during pregnancy, causalities are many times higher in the developing countries⁹. More than 300 mg of total urine proteins per 24 h and a random urine protein to creatinine ratio above 0.30 are used to diagnose preeclampsia¹¹. In 2013, proteinuria was excluded by the American College of Obstetricians and Gynaecologists as an essential criterion for the diagnosis of PE, now the GH with end-organ dysfunction is considered sufficient in diagnosis¹². At present, the aetiology of PE is not properly understood, several risk factors have been associated with the onset of condition. The serum levels of uric acid in pregnant women also indicates the chances of preeclampsia¹³. Anaemia during pregnancy, history of GH, and previous second trimester abortion are associated with increased incidence of preeclampsia¹⁴. Preeclampsia is induced by many maternal, placental, foetal factors, genetic, and environmental factors¹⁵⁻¹⁸. The hypertension associated complications increase with the number of foetuses, singletons were found 6.5%, twins 12.7%, and triplets were found to have 20% chances of complications¹⁹, females with twins have shown 14% and those with triplets have 30% chances of preeclampsia^{20,21}. Mothers with stage-1 hypertension have an increased chances of GH and PE²². Many mother associated risk factors include insulin resistance, abnormal lipid metabolism, obesity, family history, and late age during pregnancy^{23,24}.

Pathogenicity of PE has been reported as a two stage process. The blastocyst implantation into the endometrium leads to its growth into an outer trophoblast and an inner cell mass that develop outer villi and embryo respectively. Placenta is developed from the outer villi and gets attached to the mother's uterus resulting in the remodelling of spiral artery. Problems in the invasion of trophoblast to the spiral arteries result in the uteroplacental malperfusion and vascular dysfunction (stage 1)²⁵. Ischaemia-reperfusion in the placental tissue initiates the production and of reactive oxygen species (ROS), antiangiogenic factors, and cytokines and their subsequent release into the mother's circulatory system resulting in the systemic inflammation and endothelial dysfunction (stage 2)^{26,27}. All the above hypertensive disorders enhance the risk of cardiovascular and renal complications during life after pregnancy^{28,29}. GH and PE also promote the

insulin resistance by increase in the ROS production via NF- κ B-mediated pathway³⁰. The low birth weight, premature delivery or mortality, and cardiac complication in infants are also associated with GH and PE^{31,32}. Administration of aspirin can significantly (35% to 66%) reduce the chances of PE development in women with high risk of disease. Therefore, aspirin is recommended internationally to reduce the chances of PE³³⁻³⁵. Once the disease is diagnosed, delivery remains the ultimate solution for PE^{36,37}. The recent developments in aetiology, health impacts and management of GH and PE are the areas of concern for the gynaecologists in particular and for the physicians in general. In this context, the present review article was aimed to accumulative account of recent developments in GH and PE.

RISK FACTORS AND EPIDEMIOLOGY

Concept of GH and PE as two different manifestations of the same disease or separate entities with similar symptoms is not very clear. In many PE patients, hypertension occurs before proteinuria on the other hand, GH does not always progress to PE³⁸. Studies on the risk factors of GH and PE can improve the understanding of aetiological mechanisms and treatment options of these diseases³⁹⁻⁴¹. According to the hypothesis considering PE as a syndromic condition, GH and PE being the progressive stages of the same disorder, the common risk factors can suggest common aspects of aetiology⁴². Many risk factors of PE are based on the environment, drug applications, and genetics of mother and foetus. Genetics of PE is complicated, more than 178 potentially associated genes have been described. However, the mechanism of its inheritance is not clearly understood⁴³. Some of the prominent risk factors have been tabulated (Tables I, II).

Some other risk factors include maternal smoking habits, age at gestation, number of pregnancies, pre-gestation obesity, diabetes, hypertension, chronic kidney disease, multifetal pregnancies, trisomy 13, family history, and foetal genetics^{44,45}. A great deal of heterogeneity is found in the epidemiology, representative clinical characteristics, and morbidity of preeclampsia. The condition can be established after 20 weeks or after 34 weeks of gestation^{46,47}. Restriction of intrauterine growth and maternal obesity lead to early and late onset of disease. Clinically, the both types of preeclampsia are represented differently but the pattern of gene expression is similar in both cases indicating a common mechanism of maternalvascular damage⁸³. Some of the above-mentioned proteins including PAPP-A, PlGF, sFlt, PP13, and sEng are used as diagnostic markers for PE.

Table I. Gestational hypertension and PE associated genetic risk factors (genes, genetic polymorphism).

Description of risk factors		References
Candidate gene (Gene product)	Physiological role	
<i>STOX1</i> (Storkhead box 1)	Coagulation, trophoblast development, hypoxia, inflammation	48
<i>FOXD1</i> (Forkhead box D1)	Development, inflammation, immune system	49,50
<i>NLRP</i> (Nod-like receptor protein family)	Immune system, reproductive system	51
<i>TRIM28</i> (Tripartite motif containing 28)	Transcription inhibition, histone modification	52,53
<i>EPAS1</i> (Hypoxia-inducible factor 2-alpha (HIF-2α))	Regulation of physiology at different oxygen levels	54
<i>NOS3</i> (Nitric oxide synthase 3)	Production of nitric oxide, blood circulation control	55
<i>OXGR1</i> (2-oxoglutarate receptor 1)	Transcription regulation	56
<i>IGFBP1</i> (Insulin like growth factor binding protein 1)	Binds both insulin-like growth factors to enhance its half life	57
<i>MTHFR</i> (Methylenetetrahydrofolate reductase)	Re-methylation of Hcy to methionine	58-60
<i>AGT1/AGT2</i> (Angiotensinogen)	Regulation of blood pressure	61, 62
<i>AGTR1</i> (Angiotensin II receptor type 1)	Regulation of blood pressure, salts and fluids	63, 64
<i>TNF-α</i> (Tumour necrosis factor-alpha)	Multifunctional proinflammatory cytokine	65,66
<i>RGS2</i> (Regulator of G protein signaling 2)	Regulates G protein-coupled receptor signalling cascades	67,68
<i>VDR</i> (Vitamin D receptor)	Cellular response to vitamin D	69,70
<i>CTLA-4</i> (Cytotoxic t-lymphocyte antigen-4)	Immune response	71
<i>TGFB1</i> (Transforming growth factor beta 1)	Immunoregulation	72
<i>LEP</i> (Leptin)	Hormone	73,74
<i>PAPP-A</i> (Pregnancy-associated plasma protein A)	Regulates the insulin growth factor in pregnancy	75-78
<i>PLGF</i> (Placental growth factor)	Plays important role in vasculogenesis and angiogenesis	79,80
<i>sFlt</i> (Fms-like tyrosine kinase 1)	Circulating antiangiogenic protein prepared by placenta	81,82
<i>PP13</i> (Placental protein 13 produced by trophoblasts)	Vasodilation and vascularization during placenta formation	83-85
<i>sEng</i> (Soluble endoglin)	Angiogenesis	86-87

PATHOGENESIS

Although a considerable progress has been made during the last two decades yet the pathophysiology of PE is not clearly understood. The structural components and biochemical substances and processes contribute to the pathogenesis of PE. As for example, structure and physiology of placenta play a vital role in the pathogenesis of PE⁸⁴. Placenta a link between the circulatory systems of mother and foetus is a vascular organ consisting of capillaries with about 550 km in length and 15m² surface area⁹⁰. The extensive vasculogenesis process required for the normal physiology of placenta is influenced by proangiogenic and antiangiogenic factors. The concentration of circulating placental growth factor (PIGF) increases that interacts with a membrane anchored receptor known as fms-like tyrosine

kinase 1 resulting in the activation of endothelial cells and initiation of angiogenesis. During the 30 weeks of pregnancy, highest levels of circulatory PIGF are found that helps in the maturation of vessels between uterus and placenta⁹¹. On the other hand, PIGF also interacts with a soluble fms-like tyrosine kinase 1 (sFLT1) has antiangiogenic properties. It reduces the availability of PIGF to the anchored receptor, reduces nitric oxide levels, promotes the effects of proinflammatory cytokines with endothelial cells consequently resulting in the vasoconstriction^{92,93}. Hence, a balance of proangiogenic and antiangiogenic factors is necessary for the normal placental development and physiology. According to some reports the women suffering from PE have low concentrations of PIGF and higher concentrations of soluble fms-like tyrosine kinase 1 that is contrary to those with normal pregnancies^{94,95}.

Table II. Mother and foetus associated risk factors.

Risk factor	Function	References
Use of selective serotonin reuptake inhibitors (SSRIS) (Used as antidepressants, mood control, depression control)	Directly interact the transporter protein and inhibit the reuptake of serotonin / Reportedly, promote GH and PE	96,97
Assisted reproductive technology (ART) modern technology applied to treat infertility including IVF (<i>in vitro</i> fertilization), ICSI (intracytoplasmic sperm injection) etc.	Increased the chances of GH and PE	98
Elevated plasma uric acid levels (produced by the breakdown of purine nucleotides. Meat, dried beans are rich sources of purines)	Promotes GH and PE	13
Threatened miscarriage (Signs and symptoms like lower abdominal pain, little vaginal bleeding or spotting)	Enhanced the chances of GH and PE	99
High BMI (Body mass index) of mother (Higher BMI is also known as obesity)	High BMI at gestation time increases the chances of GH and PE	100
High consumption of soft drinks (carbonated drinks)	Excessive consumption of soft drinks during pregnancy increase the chances of GH and PE	101
Oocyte donated pregnancies (In case of IVF, if the female have weak egg, it can be donated by some healthy woman)	Pregnancies based on donated eggs have been associated with an increased chances of GH and PE	102
Obstructive sleep apnea (Blockage of air pathways by the relaxation of throat muscles and disturbance in breathing that results in snoring or obstruction in the sleep).	Reportedly increases GH and PE	103
Dysbiosis of gut microbiota (The disturbance in the normal (healthy) ecology of gut microbes)	Enhance the chances of GH and PE	104,105
Oxidative stress (Effect of reactive oxygen species (ROS))	Promotes the onset of GH and PE	106
Pregnancies with twins (The pregnancies with two or more foetuses)	Such pregnancies have 2 to 3 times increased risk of GH and PE development	107
COVID-19 infection (COVID-19 infection during pregnancy increases the levels of pro-inflammatory cytokines)	Chances of GH and Pe are enhanced in if pregnant women suffer from COVID-19	108

Structurally, placenta consists of many different types of cells that constitute its internal and external architecture. The inner cells that differentiate in to embryo include pericytes, endothelial cells, and smooth muscle cells. The syncytial outer layer consists of trophoblasts with an ability to invade the wall of uterine and differentiate in to the outer layer of foetus and placenta. These cells forming a thick layer are known as syncytiotrophoblasts. The surface of villi are covered by the syncytiotrophoblasts which brings these cells in direct contact with the maternal circulation^{109,110}. Preeclampsia is a human specific disease, not found in other mammalian species. This due to the high brain to body ratio of human foetus. During the third trimester of pregnancy human foetus requires up to 60% nutritional exchange from mother's circulation whereas in other mammalian species the nutritional exchange demand is about 20%¹¹¹. Therefore, the normal development and physiology of placenta is necessary to meet the nutritional requirements of foetus¹¹²⁻¹¹⁴. Pathogenesis of PE is a multi-step process mainly initiated by the abnormal placentation. Uterine arteries divide into several branches that provide blood to decidua (blastocyst

attachment area), myometrium or open as spiral arteries in the intravillous space. Invasion of defective trophoblasts into the spiral arteries cause injuries to the walls of blood vessels and result in the incomplete subsequent remodelling. This invasion may occur long before the detection of pregnancy and long before the symptoms of the disease are apparent¹¹⁵⁻¹¹⁷. Under the normal conditions the decidualization process is prolonged to the most of the areas of endometrium and increase the chances of blood supply to the foetus by remodelling of spiral arteries. However, the molecular mechanism involved in the remodelling of spiral arteries is not clearly known^{118,119}. A series of experiments involving the analysis of placental biopsy samples has shown that the spiral arteries from preeclamptic samples were significantly narrow (200 µm) as compared to those from the normal samples (500 µm). This may result in the high velocity blood flow and uterine hypoperfusion in preeclampsia¹²⁰⁻¹²².

The abnormal placentation and failure to the remodelling of spiral arteries are central to the PE pathogenesis. Studies on the analyses of placental metabolic profiles during the first trimester have revealed that energy needs remain unaffected.

Even under hypoxia, the trophoblast manage the routine proliferation by increasing the levels of HIF1 α (hypoxia-inducible factor 1 α), a transcription factor that also regulates the expression of nitric oxide (NO) synthase and vascular endothelial growth factor (VEGF)^{123,124}. Normally, the level of HIF α increases at early weeks of pregnancy and then decreases. A continuously elevated level of HIF α indicates the chances of PE development¹²⁵. The two stage model for the establishment of PE is supported by preclinical and clinical studies^{126,127}. First stage is represented by incomplete remodelling of spiral artery resulting in ischemic placenta and hypoxic conditions, in the second stage ischemic placenta releases antiangiogenic factors into the maternal circulation with subsequent endothelial dysfunction (Fig. 1).

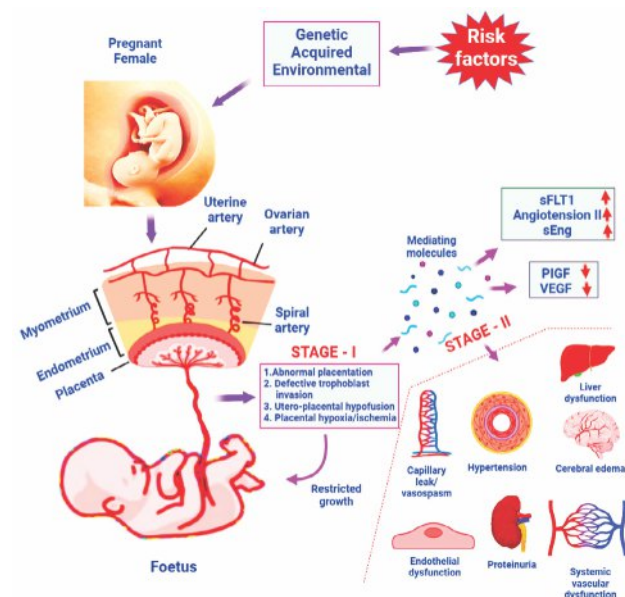


Fig. 1. Schematic representation of stage I and stage II of preeclampsia pathogenesis.

According to some studies, during spiral artery remodelling, the syncytiotrophoblast cells undergo apoptotic conditions and release a large number of syncytiotrophoblast-derived extracellular vesicles (SDEVs) into the blood circulation of mother. The level of SDEVs increase in the normal mother and becomes maximum during the third trimester. Its highest level can be found much earlier in case of PE due to hypoxic and ischemic placenta-e. A big (10% to 250%) difference of plasma SDEVs levels can be found in normal pregnancies and during PE. The difference in biological activities of these vesicles in case of normal and PE cases has been suggested^{128,129}. In fact, the SDEVs produced during PE are strong promoters of inflammation, coagulation, endothelial damage, anti-angiogenic and

vesoconstrictive conditions as compared to those produced during normal pregnancies¹³⁰⁻¹³². These processes not only enhance the injuries to the endothelial cells but also damage the placental vasculature and vessels of vital organs¹³³.

MOTHER AND FOETUS ASSOCIATED POSTPARTUM OUTCOMES OF PREECLAMPSIA

According to the growing evidence, preeclampsia is not only a pregnancy disease but the patients suffering from PE have higher risk of negative health outcomes. According to the American Heart Association the mothers with PE have a long term risk of cardiovascular diseases (CVDs), and it should be considered during CVD risk evaluation¹³⁴⁻¹³⁶. According to meta-analysis the mothers with PE history have up to 4.2 times, 2.5 times, 1.8 times, and 6.7 times increased risk of heart failure, coronary artery disease, stroke, and hypertension respectively. According to similar reports such women have a double risk for thromboembolism and 4 times risk of diabetes mellitus in future^{137,138}. The mothers with a history of PE have 3.8 times higher risk of periphery artery disease (PAD)¹³⁹, more than 30% of them suffer from asymptomatic atherosclerosis¹⁴⁰. The studies have reported increased risk of left ventricular hypertrophy or dysfunction among the mothers with PE¹⁴¹⁻¹⁴². However, the risk of all the above conditions is variable among the women from different ethnic and geographic groups¹⁴³. The individuals born after PE influenced pregnancy remain at high risk of stroke and hypertension at their adult age^{144,145}. A study conducted on 45000 individuals born to preeclamptic pregnancy has demonstrated 2.39 mmHg and 1.35 mmHg increase systolic and diastolic blood pressures, respectively. These findings suggest 8% increased risk of ischemic heart disease and 12% increased chances of stroke. The cases when the mothers had early onset of preeclampsia, the children had up to 6 mmHg increase in the overall systolic blood pressure¹⁴⁶, these individuals have a specific hypertensive vascular structure¹⁴⁷. Offsprings born after GH and PE pregnancy have problems in the cardiac structure and function¹⁴⁸. The vascular remodelling demonstrates an increased risk of neuronal damage, especially the white matter and dementia¹⁴⁹⁻¹⁵¹. An inverse relationship between the severity of GH, PE and development of foetus has been reported¹⁵².

The women with preeclampsia have high risk of diabetes postpartum. The chances of diabetes onset increases up to 2 times in the presence or absence of GE^{153,154}. Such women are prone to microalbuminuria and potential kidney disease¹⁵⁵, the risk of end stage renal disease (ESRD) was reportedly increased up to 15.5% in case of three PE pregnancies¹⁵⁶. Histopathological studies have shown focal segmental glomerulosclerosis (FSGS) associated with PE¹⁵⁷⁻¹⁵⁹. Preeclampsia associated oxidative stress, dysregulation of immune system, and growth factor has been reported to

increase the risk of neurodevelopmental problems¹⁶⁰. Studies have been conducted to associate the risk of migraine in case of PE¹⁶¹.

ASSESSMENT AND MANAGEMENT OF PREECLAMPSIA

The efficient management of PE depends on three main factors, disease prevention measures, availability of methods for early diagnosis and targeted procedures for the treatment. Gynaecologists can evaluate women for the risk factors, the high-risk individuals with pre-existing hypertension, antiphospholipid syndrome or renal disease should be advised according to established guidelines^{162,163}. Women with suspected disease onset may be subjected to placental growth factor, sFlt-1/PIGF ratio, and proteinuria evaluation¹⁶⁴⁻¹⁶⁷. Plasma levels of sEng and sFlt1 are significantly high among the women with PE as compared to those with normal pregnancy, the elevated levels can be used to determine the severity of PE¹⁶⁸⁻¹⁷². Starting before gestation to 36 weeks post gestation a moderately high dose of calcium (above 1g/day) can reduce the risk of PE¹⁷³⁻¹⁷⁵. Once PE is diagnosed, regular and continuous surveillance is recommended by urine, blood pressure test, accurate anomaly and dating scans along with the assessment of foetal growth at every 4 weeks intervals. In case of severe indications of disease plasma electrolytes, liver, kidney function tests, umbilical artery Doppler test, regular measurement of respiration rate, heart rate and blood pressure are recommended after short intervals. Medicine can be prescribed or therapeutic strategies may be adopted by the physicians after the consideration of symptoms and intensity.

In case of mild PE, the experts are recommended to prescribe fluorinated steroids before 34 weeks of gestation to promote the maturation of foetus. In case of severe PE before 34 weeks of gestation termination of pregnancy is recommended after 34 weeks of gestation immediate delivery remains the only solution¹⁷⁶. According to studies, the delayed delivery in severe PE cases can result in pulmonary edema, severe hypertension, HELLP syndrome, and liver damage to the mother. It can also cause abnormal heart rate, growth retardation, prenatal death, reverse end-diastolic flow in the umbilical artery that can be determined by Doppler ultrasound, and abruptio placentae to the foetus¹⁷⁷. Several therapeutic strategies have also been recommended to prolong safe pregnancy in case of PE, some of these strategies are being discussed here:

Antihypertensive medication is aimed to prevent hypertensive encephalopathy, intracranial haemorrhage, and pulmonary edema. There is a universal consensus that there should not be an abrupt decrease in the blood pressure to maintain utero-placental blood flow. A 10 to 20 mm of Hg decrease in 10 to 20 min is recommended by some studies^{178,179}. The medicines can be prescribed by a qualified physician, there is no clear advantage of one

medicine on the other. According to recommendations labetalol and calcium channel blockers can be used as a first line drug against hypertension. According to some other reports the use of ketanserin, nitroprusside, chlorpromazine, and diazoxide should be avoided. MgSO₄ has shown no significant effect on severe hypertension^{180,181}. The use of antihypertensive medicines during pregnancy with PE can reduce the risk of stroke but have shown hazardous impacts to the mother^{182,183}. According to a study involving 2200 individuals, the chances of PE are reduced up to 50% if a low dose aspirin is used from 12-16 weeks before gestation to 36 weeks of gestation^{173,184-186}. In another clinical study 1776 pregnant females diagnosed with low PIGF levels were enrolled, a treatment with 150 mg of aspirin per day reduced the onset of PE up to 62% as compared to placebo. On the basis of these results, low dose of aspirin is recommended to the females at high risk of PE¹⁸⁷⁻¹⁸⁹. Non-specific antioxidants have not shown significant effect in prevention of PE. The studies on the sources of oxidative stress are required to define the therapeutic targets¹⁹⁰⁻¹⁹².

Statins have been widely used for the activation of vascular function by stimulating the expression of haem oxygenase (HO), nitric oxide synthase (NO) and reducing the decrease in sFLT1 by placenta^{193,194}. These medicines have shown promising results to prevent PE in pre-clinical studies^{195,196}. Similar results have been reported in clinical studies by using pravastatin¹⁹⁷⁻¹⁹⁹. Pre-clinical studies have been conducted by using the ligands of sFLT1 such as recombinant VEGF121 for the therapy against PE in rats. The results have shown an inhibition of hypertension and amelioration against renal disease without any adverse effects to the foetus^{200,201}. Preclinical studies conducted on rodents and primates, recombinant PIGF another ligand of sFLT1 exhibited promising results against PE and hypertension. PIGF is highly specific for sFLT1 and does not bind with sFLT2 and has no adverse effects²⁰²⁻²⁰⁴. Pre-clinical studies have shown that a pregnancy associated protein known as relaxin has potential therapeutic effects against PE induced hypertension and improves utero-placental perfusion in rats²⁰⁵. Apheresis is a procedure used in medical sciences in which the blood from a person is passed through an equipment to separate a specific ingredient and the remaining blood is returned to the person's circulation. It provides an attractive option to remove antiangiogenic proteins from the blood of pregnant mothers who are at high risk of PE²⁰⁶. Studies have been conducted to remove LDL and sFLT1 from the blood of patients that give significant positive effects^{207,208}. The studies on the application of small molecules in the PE therapy have also been reported. Placental hypoxia plays a vital role in the pathogenesis of PE, the production of hypoxia induced factors i.e. HIF1, HIF2, and sFLT1 are inhibited by ouabain and digoxin resulting in the reduction of hypertension in rats with ischemic placenta²⁰⁹. Metformin is a drug that improves the sensitivity to insulin and used to treat diabetes type 2, it has been beneficial in the management of PE²¹⁰⁻²¹². Small

interfering RNA (siRNA) that are used to inhibit the synthesis of specific cellular proteins. The specific siRNA has been used to target sFLT1 in cell lines²¹³.

CONCLUSIONS

Preeclampsia, a pregnancy associated complication has serious health threat to the mother and child worldwide. The characteristic signs and symptoms of disease include onset of hypertension at before or after 34 weeks of pregnancy, release of proteins in the urine, and subsequent damage to the kidneys, liver and cerebral part of brain. There are several genetic and environmental risk factors associated with the disease. The pathophysiology of disease is not completely understood. However, a two-stage mechanism of pathogenesis is well established. According to this mechanism PE is triggered by structural and functional problems in the placenta, production of defective trophoblasts, failure in proper remodelling of spiral artery, problems in the blood flow between mother and the foetus. These conditions result in the ischemic placenta, hypoxic conditions, regulation of expression of several genes including those coding for proangiogenic and antiangiogenic factors. The consequence of all these changes from normal pregnancy conditions is the onset of PE. Disturbance in the balance between proangiogenic and antiangiogenic factors is the hallmark of PE and these factors provide biomarkers for the early detection of disease. The disease is not restricted to the pregnancy periods, it has severe postpartum consequences including CVDs, hypertension, neurological and developmental problems for the mother and child. In case of severe PE, delivery is the only treatment. However, under minor to mild conditions aspirin, antihypertensive medicines and other biochemical therapies can be adopted. Restoration of angiogenic balance during the future pregnancies by administration of proangiogenic factors, removal of antiangiogenic factors can provide a useful measure to prevent PE. However, the development of strategies against postpartum outcomes for the child and mother need extensive research.

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Authors' contribution

Conceptualization: MSN, BNM, IK. Writing original draft preparation: MSN, IU. Writing review and editing, critical revision: MNB, SJG, MMG, SIA, AU, SA.

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Effect of High Fat Diet on Liver Steatosis and Renal Function of Male and Female Wistar Rat

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) and chronic kidney disease (CKD) are the most common forms of liver and kidney disorders, respectively, characterized by the excessive accumulation of fat. This study aimed to establish a rat model for NAFLD with steatosis and renal dysfunction, having distorted nephrons, induced by a high-fat diet (HFD). A total of 30 Wistar rats, 15 males and 15 females, were randomly divided into 5 groups (n=3). The 3 experimental groups were fed with HFD i.e., ghee-based HFD, oil-based HFD, and ghee + oil-based HFD. The negative control group was given normal chow, while the positive control group was fed vegetables. At the end of the trial, rats were dissected to analyze serum biochemistry and lipid profile of the blood. The results revealed a significant increase in serum alanine aminotransferase and aspartate aminotransferase activities and creatinine and urea levels in the experimental groups. The rise in serum lipid profile parameters was observed except for high density lipoprotein in the experimental group as compared to the control group. Oxidative parameters were highest in the ghee + oil-based HFD group, indicating the highest level of reactive oxygen species in this experimental group compared to the control groups. The significant increase in organ weight was also observed in all experimental groups. The organ function of the liver and kidneys was analyzed by histological examination, which reveals accumulation of huge fat droplets in HFD-treated groups, which is associated with NAFLD and renal damage.

KEY WORDS: HFD, Obesity, Renal dysfunction, NAFLD

INTRODUCTION

Generally, a diet rich in fats, known as a high-fat diet (HFD), tends to promote the onset of metabolic syndrome¹. One of the main indicators of metabolic syndrome is obesity, which is characterized by the co-occurrence of diabetes, high blood sugar, abnormal cholesterol levels, and cardiovascular problems². In the current era, obesity is widely acknowledged as a significant and standalone contributor to the onset of numerous health conditions^{3,4}.

According to WHO, obesity is a health condition characterized by the accumulation of excessive body fat to a degree that can potentially harm one's well-being. This complex ailment results from a persistent disparity between the intake and expenditure of energy over an

extended period⁵. A recent research investigation in China, focusing on nutrition and health, has revealed a significant increase in the prevalence of obesity among the population⁶. According to another survey, global obesity rates have approximately doubled from 1980 to reach levels similar to those in 2014. Obesity has led to elevated rates of both mortality and morbidity⁷.

Several studies have demonstrated the detrimental effects of HFD on human health^{8,9,10}. There is a significant correlation between the occurrence of numerous diseases and HFD, such as NAFLD, obesity, and cardiovascular disease^{11,12,13}. Metabolic disorders, such as obesity and NAFLD, have seen an increased prevalence in various regions across the world in recent years^{14,15}. Chronic kidney disease (CKD) and NAFLD represent significant global public

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health concerns, affecting approximately 10% and 25% of the general population, respectively¹⁶. Numerous studies have demonstrated robust associations between NAFLD and both standard and non-traditional risk factors for CKD^{17,18}.

HFD enhances reactive oxygen species (ROS) in the body, which affects the body's metabolic rate. Some enzymes effectively degrade these ROS i.e., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). An elevated level of these enzymes indicates a higher level of ROS in the body¹⁹.

Non-alcoholic fatty liver disease (NAFLD) is a significant factor in the prevalence of chronic liver conditions on a global scale. It is often associated with obesity, inflammation, impaired fat tissue function, and insulin resistance²⁰. The increased likelihood of developing NAFLD and severe fibrosis is connected to the onset of obesity and insulin resistance^{21,22}. The NAFLD, which is associated with various metabolic disorders, is characterized by the accumulation of fat in the liver²³. Simple steatosis, non-alcoholic steatohepatitis (NASH), and pericellular fibrosis all fall within the range of liver conditions categorized as NAFLD. It can ultimately progress to liver cirrhosis and hepatocellular carcinoma (HCC)^{24,25}.

Studies have shown that extended consumption of HFD also alters lipid metabolism in the kidneys and results in lysosomal dysfunction, ultimately leading to renal damage in mice²⁶. The CKD is characterized by two primary indicators: diminished estimated glomerular filtration rate (eGFR) and significant proteinuria exceeding 500 mg²⁷. According to a number of recent research, a HFD may cause renal proximal tubular damage^{26,28,29} which may be linked to the activation of inflammatory pathways. These results suggest that renal proximal tubular inflammatory damage from HFD may contribute to CKD³⁰.

The examination of human diseases and the discovery of novel therapeutic targets have been greatly aided by employing mouse models^{31,32}. The purpose of the present study is to evaluate the effect of HFD on rat hematological and serum parameters. Moreover, to evaluate the organ function by feeding rats with HFD.

MATERIALS AND METHODS

Experimental animals

Thirty Wistar rats (*Rattus norvegicus*), aged 4-6 weeks old, both males and females, were purchased from the University of Veterinary and Animal Sciences and housed in animal house of Government College University, Lahore, Punjab, Pakistan, under a controlled environment (65-80°F and >40% humidity) with free availability of water and air. The normal chow typically contains carbohydrates (45-60%), proteins (14-20%), fats (4-10%), fibers (4-6%), vitamins (trace), and minerals (trace). Rats were acclimatized for 1 week and were given a normal chow diet during this period.

Preparation of HFD

HFD was prepared before the start of the trial. For the preparation of the diet, the vegetable oil and banaspati ghee (used) samples were collected in a sterile container from different regions of Lahore, Pakistan. The HFD was prepared by mixing ghee and oil in powdered chow at 30:70 ratio³³.

Experimental design

Rats (male and female) were divided into five groups each of three as G1 (negative control), G2 (positive control), G3 (experimental group I), G4 (experimental group II), and G5 (experimental group III). Negative control groups were maintained on the standard diet containing 4% fat. The positive control group rats were fed with vegetables, cereals, beans, and pulses. The G3, G4, and G5 were fed with ghee, oil, and a mixture of ghee and oil, respectively.

At the 30th day of the trial, the rats were weighed and then dissected after an overnight fasting that standardizes the metabolic state of all mice and avoids postprandial effects. Blood samples were collected by the cardiac puncture method.

Estimation of biochemical parameters

The serum was isolated after centrifugation of blood at 1000 rpm/min and stored at -80°C for biochemical analysis of liver function tests (LFTs), renal function tests (RFTs), and lipid profile. Liver and kidney tissues were also collected, fixed in formalin, and processed for histopathological examination³⁴.

Estimation of oxidative stress markers

To assess oxidative stress in Wistar rats, subjected to an HFD, catalase (CAT)¹⁹, superoxide dismutase (SOD), glutathione peroxidase (GPx)¹⁹, and glutathione reductase (GR) were estimated by following the protocol used³⁵.

Histopathological examination of liver and kidney

Fixed tissues were dehydrated in serial concentrations (30%-90%) of ethanol and then prepared for embedding in paraffin. About 5 µm-thick sections were cut and then stained with hematoxylin and eosin. Finally, the prepared slides were observed at 400X by using light the slides was observed using light microscope³⁶.

Statistical analysis

The SPSS (version 10.0) was used to process data for analysis. The data were expressed as Mean ± SEM. The comparison between groups was analyzed by one-way ANOVA following a T-test, and significance was measured at p<0.05. Alphabets were found by MINITAB version 17, which expresses significant differences. Graphs were made by Graph Pad version 5.0 and a Microsoft Excel worksheet.

RESULTS

Effect on body weight

HFD caused a significant increase in the body weight of Wistar rats (Table I). In males, the weight gain was highest in G3 fed on ghee-based HFD i.e., 122 ± 1.4 , followed by G5 fed on ghee + oil HFD and G4 fed on oil-based HFD i.e., 97 ± 0.88 and 73 ± 1.45 , respectively. The G1 and G2 showed the least weight gain compared to G3, G4, and G5.

Table 1. Effect of high-fat diet (HFD) fed for 30 days on the weight (Mean $\pm$ SEM) of male and female Wistar rats.

Groups	X	Gender	Initial body weight (g) (Day 1)	Final body weight (g) (Day 30)	Weight gain (g)
G1 (Negative control)	3	M	106 ± 3.05^c	156 ± 4.91^c	50 ± 3.17^d
	3	F	119 ± 3.05^{ab}	166 ± 2.33^c	47 ± 0.88^d
G2 (Positive control)	3	M	117 ± 2.33^{bc}	145 ± 3.60^c	27 ± 2.18^e
	3	F	107 ± 1.15^c	140 ± 2.60^d	33 ± 1.45^e
G3 (Ghee based feed)	3	M	119 ± 1.52^{ab}	241 ± 2.18^a	122 ± 1.45^a
	3	F	129 ± 3.21^a	205 ± 2.64^b	76 ± 1.15^c
G4 (Oil based feed)	3	M	126 ± 3.21^{ab}	199 ± 1.85^b	73 ± 1.45^c
	3	F	128 ± 1.15^a	236 ± 1.15^a	108 ± 1.15^a
G5 (Ghee + oil based feed)	3	M	130 ± 2.33^a	228 ± 2.90^a	97 ± 0.88^b
	3	F	113 ± 1.20^{bc}	209 ± 1.85^b	96 ± 1.15^b

The superscripts (a, b, c, d) show significant differences between means of groups ($p < 0.05$).

In female Wistar rats, the highest weight gain was observed in G4 (oil-based HFD) i.e., 108 ± 1.15 and a slight increase was observed in G3 and G5 i.e., 76 ± 1.15 , 96 ± 1.15 , respectively. While both G1 and G2 showed the least weight gain compared to experimental groups (Table I).

Table II. Effect of high-fat diet on serum LFTs of different groups of male and female Wistar rats presented in Mean $\pm$ S.E.M and their significance.

Parameters	Gender	G1	G2	G3	G4	G5	P value
ALT (μ /dL)	M	53 ± 1.15^d	46 ± 1.76^d	96 ± 1.76^b	66 ± 1.76^c	112 ± 1.45^a	0.000
	F	53 ± 1.15^d	46 ± 1.76^e	65 ± 1.15^c	98 ± 0.88^a	85 ± 1.52^b	0.002
AST (μ /dL)	M	103 ± 1.15^d	86 ± 1.76^e	205 ± 1.15^b	133 ± 1.45^c	316 ± 1.15^a	0.001
	F	106 ± 0.88^d	94 ± 1.15^e	231 ± 1.20^a	213 ± 1.45^c	222 ± 1.76^b	0.000
Albumin (g/dL)	M	4.43 ± 3.67^a	3.70 ± 3.45^b	2.56 ± 2.18^d	3.13 ± 2.98^c	2.33 ± 1.95^d	0.000
	F	4.33 ± 0.14^a	3.73 ± 0.08^b	3.13 ± 0.03^c	3.23 ± 0.08^c	3.13 ± 0.03^c	0.003
Bilirubin (mg/dL)	M	0.43 ± 0.28^a	0.33 ± 0.47^{ab}	0.16 ± 0.03^c	0.26 ± 0.12^{bc}	0.13 ± 0.01^c	0.001
	F	0.43 ± 0.03^a	0.33 ± 0.03^{ab}	0.13 ± 0.03^c	0.23 ± 0.03^{bc}	0.13 ± 0.03^c	0.000
Urea (mg/dL)	M	22 ± 0.57^d	15 ± 0.57^e	42 ± 0.57^a	28 ± 0.57^c	33 ± 0.57^b	0.000
	F	21 ± 0.57^d	17 ± 0.88^e	47 ± 0.57^a	35 ± 0.57^c	41 ± 0.88^b	0.005
Creatinine (mg/dL)	M	0.7 ± 0.05^d	0.43 ± 0.03^e	1.7 ± 0.05^a	1 ± 0.05^c	1.3 ± 0.05^b	0.005
	F	0.7 ± 0.05^d	0.4 ± 0.05^e	1.9 ± 0.05^a	1.1 ± 0.05^c	1.5 ± 0.05^b	0.001

For details of groups and statistical details, see Table I. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Effects on serum LFTs and RFTs

Table II shows in the males, the highest value (112 ± 1.45 U/l) of ALT was recorded in G5, whereas the lowest value (46 ± 1.76 U/l) was seen in G2, in male rats. In females, G4 shows the highest value (98 ± 0.88 U/l) of ALT whereas lowest value was shown by G2 (46 ± 1.76 U/l). In males, AST was highest (316 ± 1.15 U/l) in G5 and lowest (86 ± 1.76 U/l) in G2, while in females, the highest value (231 ± 1.20 U/l) was observed in G3 and the lowest (94 ± 1.15 U/l) was seen in G2.

In males, the lowest albumin value (2.33 ± 1.95 g/dL) was observed in G5 and highest value (4.43 ± 3.67 g/dL) was observed in G1. In females, both G3 and G5 showed the lowest value of albumin 3.13 ± 0.03 g/dL and G1 showed the highest value of 4.33 ± 0.14 g/dL. In males, lowest bilirubin value (0.13 ± 0.01 g/dL) was recorded in group G5 and G1 shows the highest value of 0.43 ± 0.28 g/dL. In females, both G3 and G5 showed the lowest value (0.13 ± 0.03 g/dL) of bilirubin while G1 showed the highest value; 0.43 ± 0.03 g/dL (Table II).

The highest value of urea was recorded in G3 i.e., 42 ± 0.57 mg/dL, 47 ± 0.57 and lowest value (15 ± 0.57 , 17 ± 0.88) was seen in G2 in both male and female groups, respectively. The highest value of Creatinine was seen in G3; in both male (1.7 ± 0.05) and female groups (1.9 ± 0.05). The lowest value (0.43 ± 0.03 , 0.4 ± 0.05) was seen in G2 both male and female groups, respectively.

Effect on serum lipid profile

Table III show TC and serum TG levels both increase in all HFD-treated groups. A rise in TC and TG was observed in HFD-treated groups, while their levels were normal for control groups. In males, G3 showed maximum value of TG (197 ± 1.15) and TC (290 ± 1.73); and the lowest value were

Table V. Effect of high fat diet on serum lipid profile of different groups of male and female Wistar rats presented in Mean $\pm$ S.E.M and their significance.

Parameters	Gender	G1	G2	G3	G4	G5	p-value
TC (mg/dL)	M	190 $\pm$ 2.33	136 $\pm$ 5.13	290 $\pm$ 1.73	229 $\pm$ 2.08	259 $\pm$ 2.33	0.001
	F	196 $\pm$ 1.52	175 $\pm$ 1.52	240 $\pm$ 1.73	290 $\pm$ 2.64	265 $\pm$ 1.76	0.000
TG (mg/dL)	M	145 $\pm$ 1.20	136 $\pm$ 1.45	197 $\pm$ 1.15	168 $\pm$ 0.57	186 $\pm$ 1.15	0.000
	F	143 $\pm$ 1.73	116 $\pm$ 0.88	210 $\pm$ 1.45	245 $\pm$ 1.15	230 $\pm$ 1.20	0.002
HDL (mg/dL)	M	68 $\pm$ 1.20	60 $\pm$ 0.88	31 $\pm$ 0.88	44 $\pm$ 0.88	38 $\pm$ 0.57	0.000
	F	73 $\pm$ 0.57	63 $\pm$ 1.45	47 $\pm$ 0.57	28 $\pm$ 0.57	34 $\pm$ 0.88	0.001
LDL (mg/dL)	M	94 $\pm$ 1.53	81 $\pm$ 1.29	141 $\pm$ 0.73	122 $\pm$ 2.05	134 $\pm$ 2.22	0.000
	F	95 $\pm$ 0.89	86 $\pm$ 0.69	126 $\pm$ 0.93	148 $\pm$ 1.44	135 $\pm$ 1.32	0.003
VLDL (mg/dL)	M	28 $\pm$ 0.43	22 $\pm$ 0.50	46 $\pm$ 1.01	33 $\pm$ 0.84	40 $\pm$ 0.92	0.000
	F	26 $\pm$ 1.07	16 $\pm$ 0.81	53 $\pm$ 0.88	75 $\pm$ 1.19	67 $\pm$ 0.88	0.002

For details of groups and statistical details, see Table I. TC, total cholesterol; TG, triglycerides; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

Table IV. Effect of HFD on organ weight of different groups of male and female Wistar rats presented in Mean $\pm$ S.E.M and their significance.

Organ weight	Gender	G1	G2	G3	G4	G5	p Value
Liver	M	6.3 $\pm$ 0.11 ^d	5.8 $\pm$ 0.26 ^d	12.3 $\pm$ 0.08 ^a	10.2 $\pm$ 0.10 ^b	9.5 $\pm$ 0.12 ^c	0.000
	F	5.9 $\pm$ 0.08 ^d	5.1 $\pm$ 0.06 ^e	8.2 $\pm$ 0.08 ^b	9.2 $\pm$ 0.05 ^a	7.2 $\pm$ 0.08 ^c	0.001
Kidney	M	0.62 $\pm$ 0.005 ^d	0.59 $\pm$ 0.005 ^d	1.25 $\pm$ 0.005 ^b	1.29 $\pm$ 0.008 ^a	1.06 $\pm$ 0.005 ^c	0.000
	F	0.66 $\pm$ 0.005 ^d	0.61 $\pm$ 0.005 ^e	0.95 $\pm$ 0.005 ^b	1.02 $\pm$ 0.008 ^a	0.91 $\pm$ 0.005 ^c	0.001

For details of groups and statistical details, see Table I.

seen in the G2 group i.e. 136 $\pm$ 1.435, 136 $\pm$ 5.13. In female Wistar rats, G4 exhibited the maximum values of TG and TC; (290 $\pm$ 2.64 245 $\pm$ 1.15), respectively. The lowest value was seen in the G2 group (175 $\pm$ 1.52, 116 $\pm$ 0.88), respectively. The HDL value of serum declined while VLDL and LDL rose in all HFD-fed groups. In male, G3 exhibited lowest HDL (31 $\pm$ 0.88) value and the highest value of LDL and VLDL 141 $\pm$ 0.73, 46 $\pm$ 1.01, respectively. In female Wistar rats, G4 showed the lowest value of HDL 28 $\pm$ 0.57 and the highest value of LDL 148 $\pm$ 1.44 and VLDL 75 $\pm$ 1.19, respectively (Table III).

Effect on organ weight

The normal liver tissues look dark brown while fatty liver has a yellowish-brown color. The normal and healthy liver tissue was fresh dark brown in color and light weight approx. 1.2-1.6 kg. Whereas, fatty liver was yellowish brown (Table IV), moreover, liver weight was increased in all experimental groups. In the case of male rats, the maximum liver weight was in the G3 group 12.3 $\pm$ 0.08. In the case of female rats, the maximum liver weight was in G4 group 9.2 $\pm$ 0.05.

Normal and healthy kidneys were fresh and reddish brown, with a weight of approximately 0.63 and 0.57g for male and female Wistar rats. Whereas the fatty kidneys were

yellow, and weight increased in the experimental groups. In both male and female rats, maximum weight was observed in G4 group; 1.29 $\pm$ 0.008, 1.02 $\pm$ 0.008.

Effect on oxidative stress parameters

Table V shows analyses of oxidative stress parameters showed that G5 fed with mixed HFD had the highest levels of all parameters i.e., SOD, CAT, GPx, and GR. It showed that this is the most affected group as it faced the highest level of ROS. Higher levels of ROS caused an increase in oxidative enzymes in response in both male and female rats. The positive group showed the lowest level of oxidative enzymes, which means that rats were healthier, compared to all other groups (Table V).

Histopathological analysis

Microscopic histology of the liver revealed that there was an accumulation of huge fat droplets, which were most prominent in the pericentral (centrilobular) zone, displacing the nucleus towards the periphery with bloated hepatocytes in all experimental groups. However, in positive and negative control groups, hepatocytes were almost normal, and no tissue change was observed (Fig. 1). The hepatic cords were polygonal, so having a normal shape, and no change in

cytoplasm and nucleus was observed. Sinusoids and portal area were also normal (Fig. 2).

Histological analysis of kidney tissues showed that HFD increased lipid accumulation in kidney tissues. There was deposition of huge fat droplets and glomerular injuries

between the kidney tissues in the experimental groups. While in the control groups (positive and negative), glomeruli are without any injuries. Glomerular space also remains intact (Figs. 3, 4).

Table V. Effect of HFD on oxidative enzymes of different groups of male and female Wistar rats presented in Mean $\pm$ S.E.M and their significance.

Parameters	Gender	G1	G2	G3	G4	G5	p value
SOD (U/mg protein)	M	3.7 $\pm$ 0.3 ^{cd}	2.2 $\pm$ 0.2 ^d	8.9 $\pm$ 0.6 ^b	5.2 $\pm$ 0.4 ^c	11.0 $\pm$ 0.7 ^a	<0.001
	F	2.9 $\pm$ 0.3 ^{bc}	2.2 $\pm$ 0.4 ^c	7.2 $\pm$ 0.7 ^a	4.9 $\pm$ 0.4 ^b	8.5 $\pm$ 0.8 ^a	<0.001
CAT (U/mg protein)	M	29.4 $\pm$ 0.7 ^c	21.2 $\pm$ 0.7 ^d	55.7 $\pm$ 0.9 ^b	32.4 $\pm$ 0.9 ^c	60.6 $\pm$ 1.1 ^a	<0.001
	F	26.3 $\pm$ 1.4 ^{cd}	21.2 $\pm$ 1.3 ^d	52.7 $\pm$ 1.1 ^b	30.9 $\pm$ 1.4 ^c	60.2 $\pm$ 1.7 ^a	<0.001
GPx (U/mg protein)	M	7.0 $\pm$ 0.5 ^{bc}	5.35 $\pm$ 0.5 ^c	15.1 $\pm$ 0.8 ^a	8.4 $\pm$ 0.4 ^b	16.5 $\pm$ 0.8 ^a	<0.001
	F	6.4 $\pm$ 0.4 ^{cd}	5.0 $\pm$ 0.6 ^d	12.4 $\pm$ 0.7 ^b	8.2 $\pm$ 0.5 ^c	16.0 $\pm$ 1.0 ^a	<0.001
GR (U/mg protein)	M	3.2 $\pm$ 0.4 ^c	2.8 $\pm$ 0.5 ^c	6.2 $\pm$ 0.4 ^{ab}	4.5 $\pm$ 0.4 ^{bc}	7.9 $\pm$ 0.5 ^a	<0.001
	F	3.4 $\pm$ 0.3 ^{cd}	1.9 $\pm$ 0.3 ^d	6.68 $\pm$ 0.31 ^b	4.9 $\pm$ 0.5 ^{bc}	8.6 $\pm$ 0.7 ^a	<0.001

For details of groups and statistical details, see Table I. CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase.

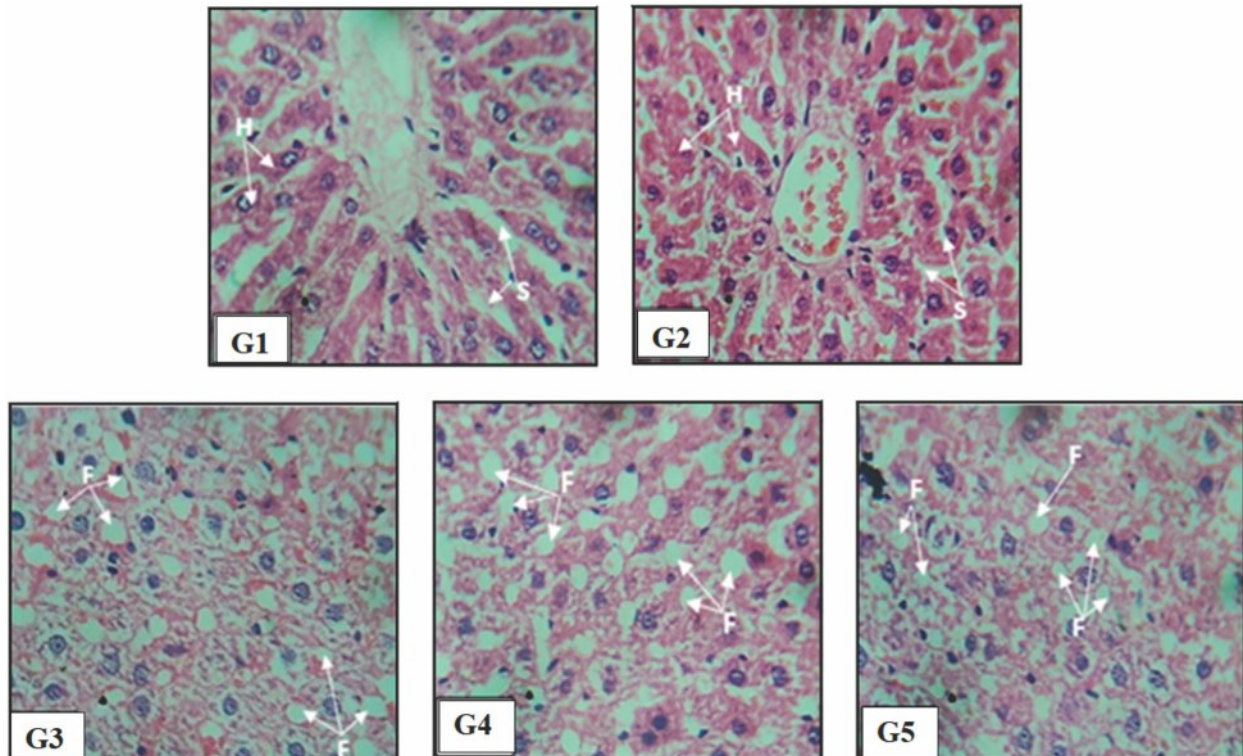


Fig. 1. Micrographs of male Wistar rats liver; G1 and G2 showing normal liver histology, H, hepatocytes; S, sinusoids, G3, G4, and G5 show histological changes in the liver with fat droplets (F) accumulation. G5 shows the highest degree of abnormal changes with distorted sinusoids and larger fat droplets. H and E staining (400x).

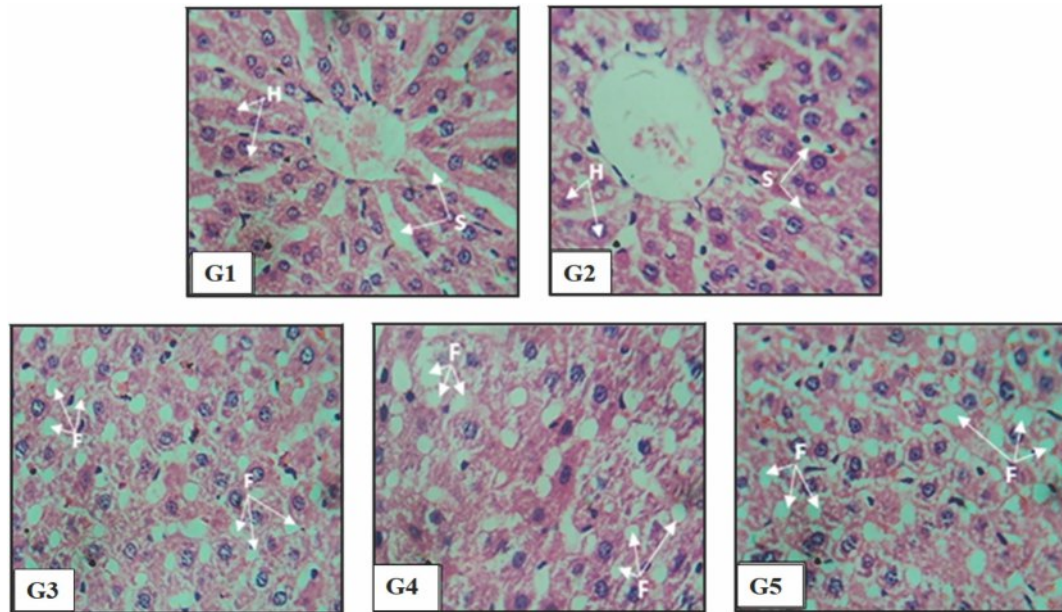


Fig. 2. Effect of high fat diet on histological structure of liver of female Wistar rats. G1 and G2 showing normal liver histology, H, hepatocytes; S, sinusoids, G3, G4, and G5 show histological changes in the liver with fat droplets (F) accumulation. G5 shows the highest degree of abnormal changes with distorted sinusoids and larger fat droplets. H and E staining (400x).

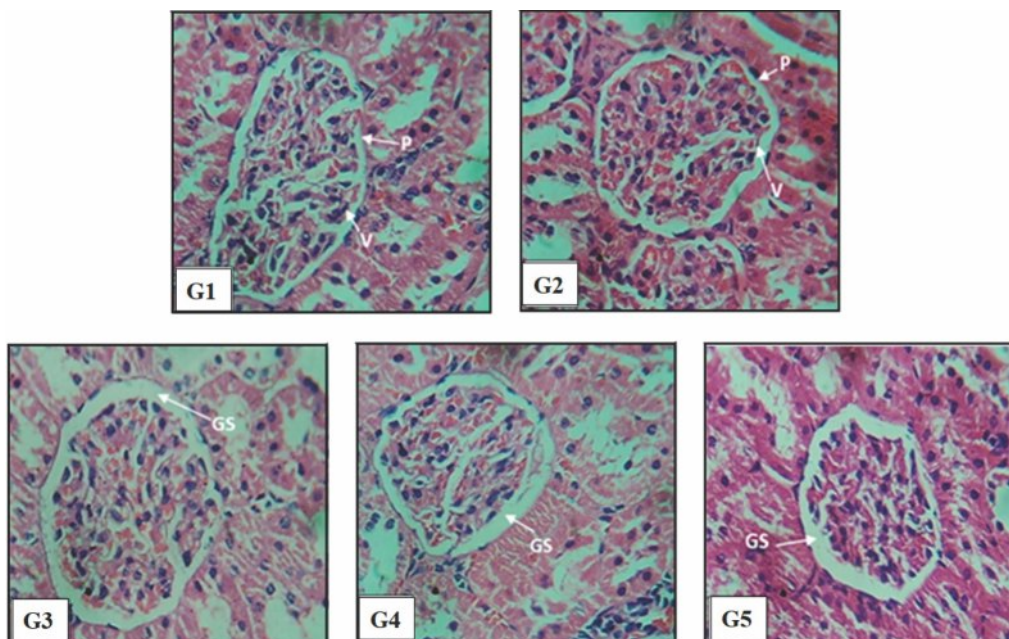


Fig. 3. Effect of high fat diet on histological structure of kidney of male Wistar rats. G1 and G2 groups show normal renal histology P, glomerular capsule; V, visceral layer of the glomerular capsule, G3, G4, and G5 show histological changes in the kidney; GS, glomerular spaces. Highest changes were observed in G5. H and E staining (400x).

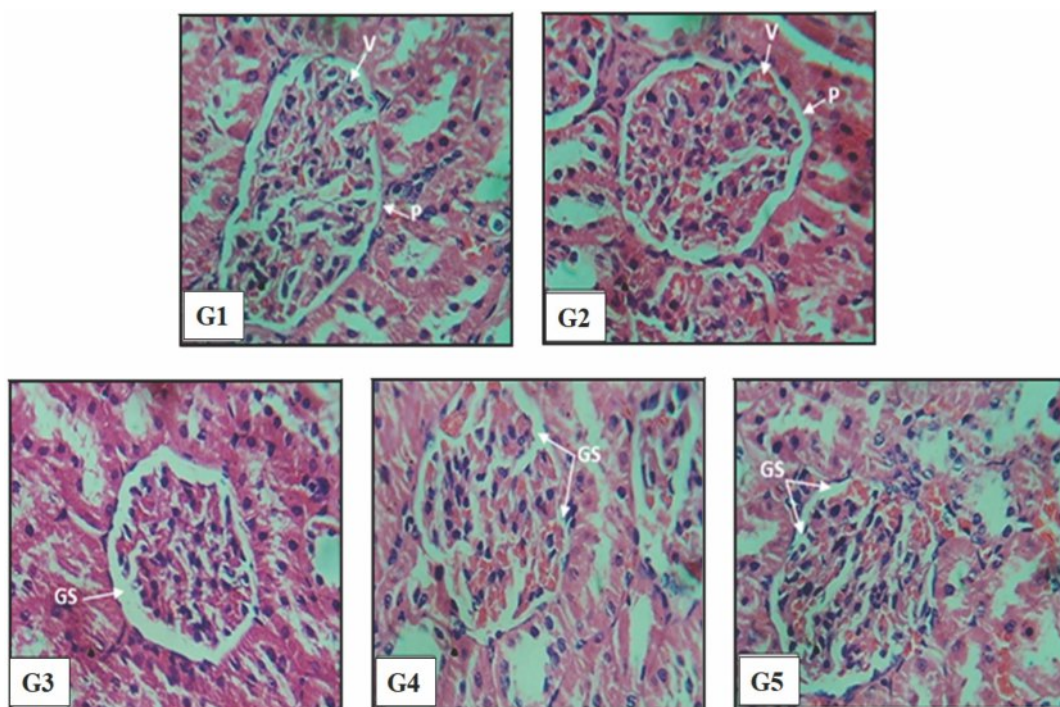


Fig. 4. Effect of high fat diet on histological structure of kidney of female Wistar rats. G1 and G2 show normal renal histology. P, glomerular capsule; V, visceral layer of the glomerular capsule; G3, G4, and G5 show histological changes in the kidney. GS, glomerular spaces. The highest changes in GS were observed in G5. H and E staining (400x).

DISCUSSION

There is a relationship between dietary fats and their effects on body mass, liver functions, and renal functions. The main objective of the study was to compare the effects of HFD and standard diet (SD) on liver and renal functions. It also reveals high fat deposition in both liver and kidneys which in turn increases the organ weight³⁷. HFD increases the serum lipid profile levels (TG, TC, LDL, and VLDL) which then affect the kidneys, liver, and its functions³⁸. HDL level declines in HFD-fed groups and rises in SD groups.

The weight of organs increased in groups fed with a HFD. The weight of the left kidney in male and female rats is higher than right. The kidney weight of female rats increased more than male rats while the male liver gained more weight than the female³⁹.

High fat deposition in the renal and liver tissues is caused by the high-fat content in the diet (dietary fats). Arteriosclerosis was the term used for the damaged arteries. Risk factors for arteriosclerosis were high cholesterol, TG levels, and high intake of saturated fats³⁷. RFTs and LFTs (BUN, CBT, ALT, and AST) increased while SAT and bilirubin levels decreased in HFD-fed groups and vice versa in the

control groups (Table I).⁴⁰ showed that rats fed with HFD for 8 weeks caused obesity in the experimental groups. The body mass increases by 10 % per week in rats. The TC and TG levels in the blood rise due to the high concentration of saturated fats in the diet. Histological study revealed that the HFD groups showed glomerular and tubular injuries. Results showed that a significant relationship existed between liver functions (ALT, Bilirubin, and AST), renal functions (Cr and U), body mass index, and triglyceride which is consistent with the statistical analysis issued^{41,42}.

This study reveals which animal model is more suited for the research on HFD effect on Wistar rats or Sprague-Dawley rats. High-fat diet added in diet of both models. Outcomes revealed that the pair influenced the body mass; but the functional results were better noticeable in Wistar rats compared to others⁴³.

The hepatic oxidative damage, steatosis, and inflammation are noticed in the rats fed with high fat which is in cahoots with the statistics of⁴⁴ in contrast to standard diet rats. The liver enzymes (ALT, AST, and ALP) are revealed to identify the hepatocyte damage. Statistics indicate that the rise in hepatic enzymes is related to liver tissue damage caused by fat deposition in the hepatocytes. The oxidative stress caused by fat eating high fats is correlated with

nephrons and hepatocyte damage. The outcomes are in agreement with the results⁴⁵.

A HFD instigates a buildup of fats and causes anomalies in both shape and function. High-fat consumption causes fat buildup and tubular injuries. The results are in cahoots with the³⁰. In renal and liver tissues, lipid profile parameters (TG, TC, LDL, and VLDL) levels rise except HDL, which is a good fat, and in diseased conditions, its level decreases. Blood levels of TG, TC, LDL, and VLDL increased due to the consumption of an HFD, while blood level of HDL decreased. Liver function enzymes (ALT and AST) and RFT levels rise in HFD groups which coincides with the results^{46,47}.

This study reveals that RFTs (blood urea nitrogen, creatinine blood test) level rises in HFD-fed groups. Our results reveal that the BUN and CBT levels increased in HFD-fed groups and standard diet (SD) groups. It then designates that the kidneys are injured in the HFD-fed groups. Histology reveals that in HFD-fed groups the tubules were structurally distorted and caused kidney dysfunctionality. HFD significantly elevates ALT and AST functionality along with the lipid and hematological profiles. These results are in good agreement with the results^{48,49}. HFDs prompt obesity. It influences liver and renal functions as well as serum lipid profiles. The current study indicates that HFD affects health by targeting organs like kidneys and liver. It causes obesity, increased cholesterol; and TG in the blood which is a determinant for arteriosclerosis. HFD causes tubular or glomerular injuries either changing their shape or influencing their function⁵⁰. Liver function enzymes (ALT, AST, and Bilirubin) and lipid profile levels elevate in the groups fed with HFD. These statistics are in cohorts with those published³⁸.

The purpose of this study was to find the cause of the weakening of renal activity and stimulating digestive diseases. In this article investigator reveals the causes of catabolic and anabolic impairment disease (high blood sugar, high blood fats, and overweight), and renal function disablement. This research caused the diseases revealed to be the intemperance of a diet containing large amounts of sugars and fats in clear-term overeating. The energy after intemperance surpasses the body's needs and it causes organ activity (especially cardiac, hepatic, and renal activity) to overexert itself which then causes disability of organs. The renal activity is certainly influenced by hyperlipidemia (High blood fats) that leads to fat buildup and shows distortion of balance among lipogenesis and lipolysis. It causes alterations in the shape and activity of organs that progress into nephron injuries. The treatment for long-term renal disorder is considered to be reducing the intemperance of sugar and fats⁵¹.

In this study it was noted that G3 fed with mixed HFD had increased levels CAT, SOD, and GPx enzymes as compared to the control groups. Similar findings were noted in a study reported⁵². In another study Liebert and Murias⁵³,

reported that HFD can cause increase in enzyme GR in rats which resembles with present study. Increased antioxidant enzyme level indicates that HFD has induced an increase in ROS in the body and the body has responded by elevating the antioxidant level.

Being overweight was the cause of the long-term renal disorder but the process was difficult to find. In this research, the investigator reveals that a diet containing high fats instigates kidney injury in animal models (mice). The animal model feeding on a diet containing high fats for 4 whole months had progression of over mass index, high blood sugar, and renal deterioration indicated by high albumin in urine and buildup of UN and Cr in serum. The damage of glomeruli and tubules was noticed³. Our study showed similar results. The damage to the glomeruli and tubules in the kidneys was evident and showed structural alterations. High albumin in urine (albuminuria), obesity, and hyperlipidemia in tissue (fat deposition) were noticed in the histopathology of renal tissues.

In the late decennium rising in commonness of long-term renal disorder and the over-weight index had developed. Fat poisoning in the kidneys is linked with being overweight. The buildup of fat in renal tissues was the source of deterioration and kidney scarring. If the fat buildup surpasses the amount the body can accumulate then fatty acids leak into the blood and then accumulate in various other organs including renal organs. This study aimed to reveal that fat poisoning causes alterations in kidney physiology and its activity⁵⁴.

CONCLUSION

Our results exhibited a significant increase in the weight of HFD-fed Wistar rats. Serum LFTs and RFTs parameters were elevated which indicated hepatic and renal injury. Hematological parameters showed significant alterations and the serum lipid profile has higher values except HDL. The rise in TG and TC levels indicated the arteriosclerosis and tubular injuries in renal tissues. Fat deposition in the liver caused steatosis while in renal tissues caused nephron damage. Histology of liver showed inter-hepatocytic accumulation of huge fat droplets. Renal histology also indicated fat deposition in glomeruli and tubules (nephrons) in HFD-fed groups in contrast to control groups.

DECLARATIONS

Authors' contribution

DAB conceived and supervised the study. AR edited the manuscript. SM, KM, Arifullah, and AK contributed to the experiments and wrote the manuscript.

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Generative AI and AI-assisted technology statement

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Statement of conflict of interest

The authors have declared no conflict of interest.

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Prevalence of MDR and XDR in Clinically Relevant Enterobacteriaceae from Local Food Samples

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ABSTRACT

In this study, Enterobacteriaceae were isolated from food samples, either in raw or cooked form, including some bakery items, meat, fruits, vegetables, and sweets. This study aimed to obtain multidrug (MDR) and extremely drug-resistant (XDR) bacteria that have a high potential for biofilm formation and confer appreciable resistance to heavy metals, as foodborne microbes are becoming a menace even in the modern medicine era. In the current study, different raw and cooked food samples were evaluated for the prevalence of Enterobacteriaceae. A total of 40 food samples were used, and 33 bacterial isolates were assessed for their antimicrobial resistance potential, from which 7 isolates were later selected to determine their metal resistance and biofilm production profile. Out of 7 selected bacteria, 4 were XDR and 3 were categorized as MDR. All bacterial isolates were resistant to colistin, amikacin, gentamicin, cefexime, tetracycline, and sulphonamides. Among carbapenems, 50% of the isolates were resistant to meropenem, and 16% of the isolates were resistant to imipenem. The potential XDR bacteria were isolated from meat and milk, and fish and capsicum were also found as the main reservoirs of MDR bacteria. Ceftazidime-avibactam was found as a remarkable antibiotic, as all of the bacteria were sensitive to it. Nearly 80% of the isolates were sensitive to imipenem and levofloxacin. It was seen that those isolates that had high resistance against antibiotics had high minimum inhibitory concentration (MIC). Similarly, biofilm formation capacity also contributes greatly to developing mechanisms to resist antimicrobial drug penetration through the cell membrane. So, the majority of bacteria were biofilm producers and XDRs. Combined with it, the heavy metal resistance and biofilm-producing potential make the situation more challenging. There is a dire need to combat MDR and XDR pathogens to make the treatment of infectious diseases a complete reality and to call modern medicine an unchallengeable weapon.

KEY WORDS: Foodborne enterobacteriaceae, Multi-drug resistance, Extreme-drug resistance, Antimicrobial drugs, Metal sensitivity, Biofilm

INTRODUCTION

Foods originating from animals, such as milk, beef, and chicken flesh, are rich in proteins, which are vital for the growth and development of the body. Animal-based foods, however, have the potential to act as a carrier and medium for a variety of germs that can lead to illness, sickness, and even death. Foodborne infections are starting to pose a threat to public health worldwide¹. Drug resistance in bacteria has escalated to risk-taking levels and poses a major problem in therapies for different diseases. The repercussive impacts of

this obstacle are broadened life losses as well as putting a price on health maintenance². Drug resistance becomes a menace when it comes to the treatment of chronic diseases. If any drug shows inefficacy, a constraint is put on treatment, as every patient may show different pharmacokinetics. Costly drugs and higher doses don't necessarily lead to a better response. Higher doses given to have early recovery pave the road to side effects as well as drug resistance³.

Multidrug-resistant (MDR) bacteria are those that have insusceptibility to more than one drug, or if bacteria are not susceptible to at least one sub-category of two or

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three classes of antibiotics. Extremely drug-resistant (XDR) bacteria are defined as those bacteria that show resistance to one sub-category in all antibiotic classes, or bacteria that are only sensitive to one or two classes. Pandrug-resistant bacteria are those that show no sensitivity to any sub-category in all classes of antibiotics⁴. Enterobacteriaceae are Gram-negative pathogens residing in the gut flora and intestines of humans and other animals. They can also reside in the soil or water. Common examples include *Escherichia coli*, *Salmonella*, *Shigella*, *Yersinia*, *Proteus*, *Klebsiella*, *Citrobacter*, *Providencia* etc.⁵. Dairy products and meat have always been a center of concern for people around the globe. These sources are thought to be contaminated with Enterobacteriaceae. Indeed, they are used as a marker for evaluating the food quality. Therefore, the assessment of food quality is fundamental to prevent risks associated with food consumption⁶.

To diagnose infections associated with *Enterobacter*, the involvement of the cultures is mandatory. To evaluate blood samples, two sets are used: One for the aerobic bottle and another for the anaerobic bottle. Urinalysis can also be performed. MacConkey agar is used for the indication of fermentation, and the indole test is crucial to differentiate between different species⁷. However, *Enterobacter* can be recovered from sputum samples for respiratory diseases, surgical wounds, and blood⁸. One of the weapons used by Enterobacteriaceae for its pathogenicity is its drug resistance. Beta-lactamase is a major agent for evaluating it. It can hydrolyze the beta-lactam ring in different drugs-approaching drug resistance. Talking about Enterobacteriaceae, *E. coli* is a key member. Usually, it is present as a commensal in the digestive tract, but due to certain virulence genes and antibiotic resistance genes, it can cause many infections, including digestive tract diseases, urinary system dysregulation, as well as inflammatory reactions in the brain, etc⁹.

Among the Enterobacteriaceae family, the genus *Enterobacter* is highly associated with infections. Not all *Enterobacter* species cause infections; some of them lead to infections in the hospital environment; some are associated with community-borne infections, including airway infections, urinary and cystitis, cellulitis, heart and long bones problems, catheter-associated septicity, nosocomial sinusitis, etc. One of the weapons used by Enterobacteriaceae for its pathogenicity is its drug resistance. β -lactamase is a major agent for evaluating it. It can hydrolyze the beta-lactam ring in different drugs-approaching 'drug resistance'¹⁰.

Dairy products and meat have always been a center of concern for people around the globe. These sources are thought to be contaminated with Enterobacteriaceae. Indeed, they are used as a marker for evaluating the food quality. Therefore, the assessment of food quality is fundamental to prevent risks associated with food consumption⁶. The reason behind their efficiency to assess food quality with greater scope as compared to coliforms is because of one of the

remarkable features i.e., resistance to the environment. So, Enterobacteriaceae have been used as a crucial element to gauge the sanitation of food and dairy products.

Biofilm aids bacteria in shielding bacteria from chemicals, protects bacteria from stressful conditions during food processing, and protects them from disinfectants¹¹. It was seen that patients having bacteremia, septicemia, ulcers, and diabetes are more prone to MDR and XDRs, and these strains are also exhibiting biofilms. Also, members of Enterobacteriaceae family have membrane vesicles (MVs)¹². These vesicles are coated with lipids, and they help the members of the biofilm to interact with each other for biofilm production. These vesicles have proved to be the agents for virulence and toxicity and perform their job in causing host infections¹³. To combat biofilm-producing capability, it is very crucial to check for its potential to show antibiotic resistance. Those bacteria that have the attribute of biofilm production are lost prone to the stresses imposed by the environment or even by the drugs. Biofilm producers are 10^3 times less prone to drug therapy as compared to non-producers¹⁴. Biofilms assist in drug resistance by acting as a permeability barrier to drugs, degrading enzymatic proteins, and drug-excluding capabilities¹⁵.

Novel antibiotic discovery efforts are in progress. For some bacteria, there were very less treatment options i.e., *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* Antimicrobial resistance poses a considerable challenge to even modern medicine. Pressurized by the regulatory bodies, scientists are having more work to get some new antibiotics to treat havoc-creating pathogens, including MDR and XDR bacteria¹⁶.

Keeping in view the clinical implications of difficult-to-treat pathogens, it is foremost to develop antibiotics for such bacteria. Carbapenem resistance may occur due to membrane porins and efflux pumps. Aminoglycoside resistance may occur due to transposon elements harbored by bacteria. Fosfomycin exhibits the potential to inhibit cell wall synthesis, but it is not used for MDR infections. Ceftazidime-avibactam is used to treat abdominal infections, UTIs, and hospital-acquired infections that were very difficult to treat previously¹⁷. Avibactam prevents ceftazidime from degradation and ensures its longevity. Meropenem alone can confer no antibacterial activity against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, but when combined with a non- β -lactam- β -lactamase inhibitor can assure a good activity. Although vaborbactam is not antibacterial but it can restore the role of meropenem by protecting it from degradation¹⁸.

MATERIALS AND METHODS

Sampling and isolation of bacteria

For the isolation of bacteria, different food samples were used, either from local vendors, private label brands, or confectioneries (see Table I).

Isolation of Enterobacteriaceae

To specifically isolate Gram-negative bacteria, MacConkey agar (Peptone 17g, proteose peptone 3g, lactose monohydrate 10g, bile salts 1.5g, sodium chloride 5g, neutral red 0.03g, crystal violet 0.001g, agar 13.5g, 1000 mL distilled water) was used¹⁹. Food samples were first diluted in normal saline and then spread on poured MacConkey agar plates. MacConkey agar was autoclaved, and agar plates were prepared. Then, 50 μ L of the prepared food sample was dispensed in the center of prepared plates with the help of a micropipette, and the food preparations were spread with the help of a sterile glass spreader sunken in ethanol. Even distribution of the suspension was ensured throughout the surface of the poured agar plates. The plates were placed on the bench top for 10 min to ensure dryness and proper absorbance²⁰.

After the absorption of the suspension into the agar plates, the plates were inverted and placed in an incubator at 37°C for 24 h. After 24 h of incubation, plates were analyzed for growth. Colonies were counted semi-quantitatively, and from each plate, two well-isolated and different colonies were streaked on MacConkey agar plates and incubated for 18-24 h at 37°C.

Preparation of minimal salt medium

Minimal salt medium (MSM) (Glucose 0.25g, MgSO_4 0.01g, CaCl_2 0.013g, KH_2PO_4 1.25g, Na_2HPO_4 0.175g, $(\text{NH}_4)_2\text{SO}_4$ 0.25g, FeSO_4 0.335g, and agar 3.75g were added up to 500 mL distilled water) was used for the growth of microorganisms in the absence of carbon source to evaluate growth upon stress conditions in the presence of heavy metals²¹. However, glucose was added to check if the organism relies upon salts after the complete utilization of glucose.

Kirby-Bauer method to evaluate antimicrobial potential of bacteria

Susceptibility tests were used to evaluate the antimicrobial potential of bacteria in the laboratory environment. Disc-diffusion (Kirby-Bauer technique) was used to determine the sensitivity or resistance of bacteria to antibiotic drugs²².

Mueller Hinton (MH) agar (Beef infusion solids 2g, starch 1.5g, casein hydrolysate 17g, agar 17.5g, 1000 mL of distilled water, Final pH 7.3 $\pm$ 0.2 at 25°C) was prepared and autoclaved under standardized conditions and poured into plates. The plates were allowed to solidify. Bacterial cultures grown in L-broth and swabbed on MH-agar plates after adjustment with a 0.5 McFarland standard²³. Then, the following antibiotics were applied. Ciprofloxacin, gentamicin, cefepime, meropenem, tazobactam, tetracycline, sulphonamide, colistin, amikacin, levofloxacin, imipenem, aztreonam, ceftazidime-avibactam. Plates were incubated at 37°C for 24 h. After which zones of inhibition

were measured with the ruler to measure the diameter around the disc. The size of zones of inhibition was measured in millimeters and compared with the Clinical and Laboratory Standards Institute (CLSI). Bacteria were categorized as resistant, intermediate, or sensitive with CLSI guidelines. MDR and XDR bacteria were identified.

Biochemical characterization

For biochemical characterization, catalase, oxidase, triple sugar iron, urease, indole, methyl red/Voges-Proskauer, and citrate tests were performed²⁴. Gram staining was done to isolate Gram-negative rods.

Molecular identification

The phenol-chloroform-alcohol method was used for DNA isolation²⁵, which was then used for amplification of 16S rRNA using the following primers

27F= 5'AGAGTTTGATCCTGGCTCAG3' and
1492R= 5'GGTTACCTTGTTACGACTT3'.

The PCR reaction mixture (50 μ L) comprised 2.5 mM MgCl_2 , 1 μ L of 10X Taq buffer with KCl (100 mM Tris-HCl pH 8.8, and 500 mM KCl), 200 mM deoxyribonucleotide triphosphate (dNTPs), 50 pmoles of each forward and reverse primers, 1 unit of Taq DNA polymerase and 15 μ L of DNA (20 ng/ μ L DNA conc) while the thermal cycle consisted of initial denaturation at 95°C for 2 min, followed by 40 cycles each of denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72 °C for 1 min. The final extension was done at 72°C for 10 min. The PCR product was visualized on 1% agarose gel and then purified using the Mo-Bio Laboratories purification kit 16S rRNA PCR product. The purified PCR product was sent for sequencing to Macrogen, Korea, and the sequence obtained was then aligned by utilizing BLAST analysis. Moreover, a dendrogram was created by using the MEGA7 program on the basis of homology.

Metal resistance activity

To check metal resistance or minimum inhibitory concentration (MIC), MSM was used. Minimal salt media were prepared and autoclaved as per standard conditions. Five metals i.e., cadmium (CdCl_2), arsenic (NaAsO_2), lead (PbNO_3), chromium ($\text{K}_2\text{Cr}_2\text{O}_7$), and zinc ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), were used. Firstly, a stock of 10 mM of each metal was made, and then working concentrations of 0.5 mM, 1 mM, 3 mM, 5 mM, and 7 mM were prepared. For each strain (MT st1, MT st2, MK, SM st2, GP, CO st1, and SP; isolated from food samples), five plates using MSM were prepared. After pouring, the media was allowed to solidify. Then, quadrant streaking was done on MSM agar plates. Plates were incubated at 37°C for 24 h and growth was noted.

Biofilm production test

Congo-red media was prepared (Glucose 60g, Brain

Heart Infusion agar 11.1g, Congo red 0.24g, agar 6g, and added to 300 mL of distilled water²⁶ and autoclaved, poured into autoclaved Petri plates, and inoculated with bacterial strains by the quadrant streaking method. The plates were incubated at 37°C for 24 h. Results were visualized as crystal-textured black colonies, which serve as a qualitative indicator of biofilm production²⁷.

RESULTS AND DISCUSSION

Table I gives an overview of bacterial prevalence in raw and cooked foods. It was found that raw chicken, raw milk, carrot, green chilli, spinach, mutton, fish, and grapes had enormous bacterial communities from the raw food group, whereas corn, sugarcane, brinjal, banana, and potato showed negligible, and ketchup, egg shell, black pepper, pomegranate, peas, and onion showed no bacterial growth. Among the cooked foods, tremendous bacteria were obtained from Kabab and chestnut, whereas other samples like boiled chicken, fried eggs, pizza, snacks, and sauces showed no growth.

From a total of 40 food samples, almost 69-70 strains were isolated. Based on the antimicrobial resistance (MDR and XDR profile), 11 strains were selected on which further antimicrobial resistance was gauged using five more antibiotics i.e., colistin, amikacin, levofloxacin, Imipenem, and aztreonam. Out of these 11 strains, 1 strain was selected for 16S rRNA sequencing, and it was designated as extremely drug-resistant bacteria (XDR), having accession number PP978759.

Bacterial identification

Various biochemical tests were performed to identify the bacteria. The partial 16S rRNA gene was amplified with general primers, and bands appeared in parallel with a 1500bp ladder sequence. The phylogenetic tree was also constructed based on 16S rRNA sequencing (Fig. 1). It was found that the query sequence was of *Escherichia coli*, having the accession number PP978759.

Antibiotic resistance profile

It was found that the subject strains were highly resistant to ciprofloxacin and less resistant to levofloxacin, which both belong to the fluoroquinolone. There was a high resistance shown against gentamicin and amikacin by all strains, which both belong to the aminoglycosides. Among carbapenems, 50% of strains were resistant to meropenem and 16% of strains were resistant to imipenem. Among cephalosporins, 100% of strains were resistant to cefexime. For tetracyclines, 100% of strains were resistant to tetracycline. 16% of strains were resistant to aztreonam, and 33% of strains were resistant to ceftazidime-avibactam. Similarly, almost 70% of strains were resistant to sulphonamides. For bacterial infections of the urinary system,

Table I. Semi-quantitative assessment of bacterial prevalence in raw and cooked foods.

Raw foods	Growth
Raw chicken	++
Raw milk	+++
Banana	+
Ketchup	No growth
Egg shell	No growth
Black pepper	No growth
Rice	No growth
Carrot	+++
Potato	+
Spinach	+++
Mutton	+++ (<i>E. coli</i> isolated)
Phallian	+++
Fish	+++
Grapes	++
Grapefruit	+
Pomegranate	No growth
Peas	No growth
Onion	No growth
Brinjal	+
Green chilli	+++
Beef	++
Sugarcane	+
Fenugreek	++
Corn	+
Apple	No growth
Capsicum	+
Coconut	+++
Paan	+++
Cooked Food	Growth
Fried egg	-
Pizza	-
Sweets	-
Salan	-
Boiled chicken	-
Samosa	-
Macaroni	-
Water chestnut	+++
Chapati	-
Naan	-
Kebab	++
Snacks	-
Fruit chat	-
Sauces	-
Biscuit	-

+++ , Enormous growth; ++, Good growth; +, Less growth.

sulphonamides and fluoroquinolones can be used, but in this research, 100% strains were resistant to ciprofloxacin. The antimicrobial resistance profile of *E. coli*, a potential isolate from the mutton sample, elucidated as being resistant to ciprofloxacin, meropenem, tazobactam, sulphonamide, tetracycline, colistin, levofloxacin, imipenem, and aztreonam, and was sensitive to gentamicin, cefexime, and amikacin. It was categorized as XDR as it was resistant to one sub-category in all antimicrobial classes.

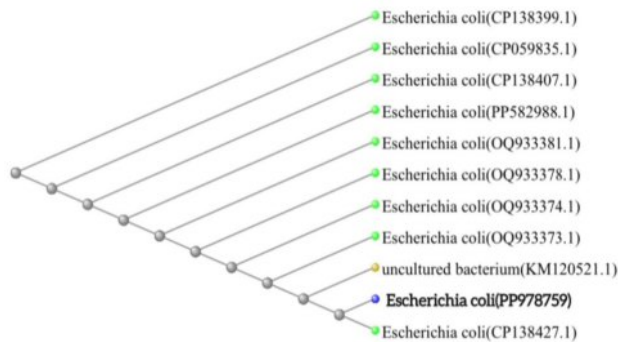


Fig. 1. Sequencing-based phylogeny obtained through 16S rRNA sequencing.

It was found that the subject strains were highly resistant to ciprofloxacin and least resistant to levofloxacin, which both belong to the fluoroquinolone. A high resistance pattern was shown by all strains against gentamicin and amikacin, which belong to the aminoglycosides. Among carbapenems, 50% of strains were resistant to meropenem and 16% of strains were resistant to imipenem. Among cephalosporins, 100% of strains were resistant to cefexime. For tetracyclines, 100% of strains were resistant to tetracycline. The aztreonam was resistant to 16% of the strains, and 33% of the strains were resistant to ceftazidime-avibactam. Similarly, almost 70% of strains were resistant to sulphonamides (Fig. 2).

Among the different food samples used, pathogens from mutton were thought to be highly resistant to almost all antibiotics, hence being XDR. Similarly, the pathogen extracted from milk was also found to be heavily resistant to ciprofloxacin, gentamicin, cefexime, meropenem, tazobactam, sulphonamides, amikacin, and aztreonam and sensitive to levofloxacin, imipenem, and ceftazidime-avibactam. The pathogen isolated from grapes was found to be resistant to all antibiotics mentioned but sensitive to meropenem, levofloxacin, imipenem, and ceftazidime-avibactam. Similarly, the pathogen isolated from fish was resistant to aminoglycosides, cephalosporins but sensitive to carbapenem and aztreonam. The pathogen isolated from capsicum was only sensitive to imipenem, hence termed as XDR.

Among the studied strains, 66% strains were XDR and 33% of the strains were MDR. Among the studied strains, 66% strains were XDR and 33% of the strains were MDR. The figure describes well the healthcare burden imposed by the heavy isolation of Enterobacteriaceae from different departments of the hospital, from the study done in 2016. 79% of *E. coli* strains were MDR, and 22% of them were XDR. Similarly, out of a total of 212 strains, 66% of *Pseudomonas aeruginosa* were MDR and 29% of them were XDR⁴. In the current study, XDRs were only found in mutton, milk, and capsicum samples, while bacteria in fish and grape samples were MDR (Fig. 3).

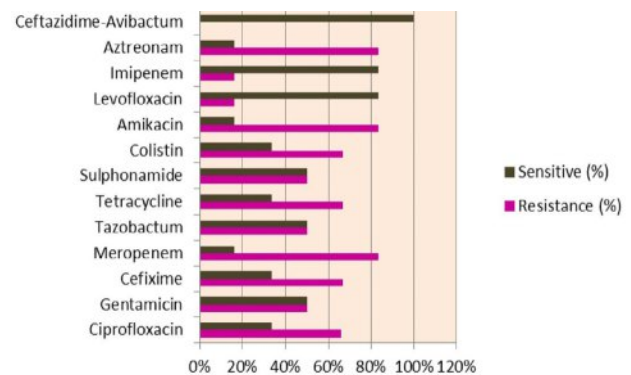


Fig. 2. Bacterial sensitivity and resistance (%) against the antibiotics used.

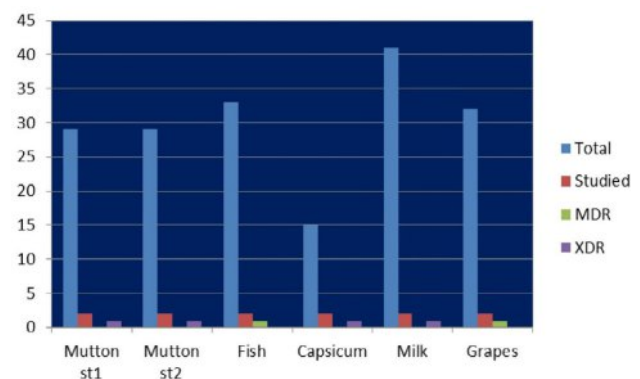


Fig. 3. Incidence of MDR and XDR in different food samples containing *E. coli*.

Widely distributed MDR bacteria threaten animal and human health. According to the study done²⁸ in China, 1,468 (97.54%) *Escherichia coli* strains were isolated from 1,505 pig (1,060) and chicken (445) anal swab samples. These isolates had a high resistance to tetracycline (92.92%), sulfisoxazole (93.05%), florfenicol (83.11%), and ampicillin

(78.27%). More than 88.68% of the strains were MDR bacteria. A low AMR ratio to the last-resort antimicrobials tigecycline (0.75%), colistin (1.36%), and meropenem (0.75%) was found. The AMR of *E. coli* from pigs was higher than that of chickens. For biofilm production, 83% of the isolates had the potential for biofilm formation. According to the study done in 2022 in Egypt of different food samples from different markets, it was found that out of a total of 30 strains, 25 strains of Enterobacteriaceae were having the ability to form the strongest biofilm, and 5 strains were late biofilm-formers²⁹. However, in this current research, it was seen that *E. coli* was found in 90% of raw food samples, and the most frequent one was mutton.

Metal resistance profile

For metal resistance activity, out of 7 isolates, one strain was sensitive to all metals at all concentrations. The rest of the isolates were resistant to all metals, at specific MIC. Isolates MK, MT st1, GP, and SP resisted all concentrations of cadmium. MK, GP, SM, and SP resisted all concentrations of arsenic. MT st1, MK, and GP resisted all concentrations of Zinc. The SM and SP were moderately resistant to arsenic, lead, chromium, and zinc. MT st1 was also weakly resistant to arsenic and lead but strongly resistant to zinc and sensitive to chromium. *E. coli* was resistant to cadmium and zinc at all working concentrations. MIC for arsenic and lead was 3 mM, whereas it was entirely sensitive to chromium.

According to the study done in Brazil in 2022, MDR Enterobacteriaceae were highly resistant to zinc, copper, chromium, and mercury, with varying minimum inhibitory concentrations. It was found that for MDR, the MIC was higher for toxic metals as compared to those bacteria that didn't confer any resistance to antibiotics. For the latter group, MIC was lower³⁰. According to the study done on chicken meat in Dhaka in Bangladesh in 2022, all strains (n=12) formed biofilms and were very highly resistant to ciprofloxacin, levofloxacin, ampicillin, meropenem, and colistin. These bacteria were highly resistant to zinc, copper, and nickel. It was found that the resistance to copper and zinc was interrelated with antibiotic resistance³¹.

MT ST1 was later sequenced and identified as *E. coli*, one of the potent pathogens isolated from the mutton sample.

Biofilm formation

For biofilm production, 83% of the strains had the potential for biofilm formation. Samples isolated from mutton, milk, and grapes were biofilm producers, whereas the bacterial isolate from capsicum was not a biofilm producer. Interestingly, isolates from mutton and milk were XDR, emphasizing the combating potential to resist antibiotics, but isolates from grapes were MDR. Although an isolated bacterium from capsicum was not a biofilm producer, it was XDR, ruling out that there is not always a combined potential (Fig. 4).



Fig. 4. Biofilm production by a bacterium (Left); Negative control (Right).

CONCLUSION

The study concludes that MDR and XDR isolates were prevalent in different food samples in a certain ratio. Also, there is an association between antibiotic resistance and metal resistance, but it is not a universal law. It was found that among many strains isolated, *Escherichia coli* was extremely drug resistant, but it was sensitive to 3 out of 5 metals used. When it comes to the prevalence of XDR isolates alone, 50% (2 out of 4) of XDR isolates were obtained from mutton; 50% (2 out of 4) of XDR isolates were collected from milk and capsicum. Talking about MDR prevalence alone, 50% of MDR isolates were obtained from fish, and 50% of MDR isolates were acquired from grapes. Collectively, 66% of isolates were XDR, with mutton being the source of XDR on the edge, whereas 33% of isolates were MDR. All isolates had the likelihood of producing biofilms except capsicum, although it was an XDR isolate. To wrap things out, this study concludes that there is a strong association between antimicrobial resistance, metal resistance, and biofilm production, but it is not true in all cases, as many of the bacteria have acquired different resistance mechanisms and many bacteria do not possess such mechanisms which render them sensitive to pressures applied.

DECLARATIONS

Authors' contribution

AR and DAB conceived the idea and edited the manuscript. IAN and EA contributed in the experiments and wrote the manuscript.

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Generative AI and AI-assisted technology statement

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Statement of conflict of interest

The authors have declared no conflict of interest.

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Repositioning of FDA Approved NSAIDs for Potential STAT3 Inhibitors: A Molecular Docking Study

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ABSTRACT

Signal transducer and activator of transcription 3 is a latent transcription factor involved in various cellular processes, cell proliferation, apoptosis, inflammation, immunity, angiogenesis, and metastasis. Controlled regulation of STAT3 activity is maintained in normal cells, but its sustained activation causes tumor growth and carcinogenesis. This computational study aims to elucidate the potential of FDA-approved non-steroidal anti-inflammatory drugs in inhibiting STAT3. Molecular Docking was performed using AutoDock and visualized through LigPlot+ and Biovia Discovery Studio Visualizer. The findings demonstrate that NSAIDs, including ibuprofen, naproxen, diclofenac, celecoxib, mefenamic acid, etoricoxib, indomethacin, and aspirin, exhibit substantial binding affinity with STAT3 monomers. The binding energies calculated are -5.6 to 7.5 kcal/mol, with inhibition constants from 7.5 to 3.03 μ M, respectively. Notably, key amino acid residues such as GLN326, HIS457, LYS244, PRO487, ARG335, ASP566, CYS468, PRO471, LYS383, LEU378, VAL323, THR456, LYS318, GLU455, ASP570, LYS573, CYS251, TRP474, PRO336, ASP334, SER403, ILE258, ARG325, LYS348, ARG350, and GLU324 are identified as pivotal in mediating the interaction between NSAIDs and STAT3. This study provides valuable insights into the molecular interactions between FDA-approved NSAIDs and STAT3, offering a foundation for further exploration of their potential as therapeutic agents in the context of STAT3-mediated diseases.

KEY WORDS: FDA-approved drugs, Molecular docking, NSAIDs, STAT3, Anticancer, Drug discovery

INTRODUCTION

Signal transducer and activator of transcription 3 (STAT3) is a member of the cytoplasmic transcription factor family known for signal transduction and activation of transcription, which includes seven members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6¹. STAT1 has structural similarity to STAT3 and STAT5, and primarily functions as an oncogene². Particularly, STAT3 is a DNA-binding protein that ultimately regulates the essential genes like cell proliferation, metastasis, differentiation, apoptosis, immune responses, angiogenesis, nephrogenesis, gliogenesis, inflammation, and hepatogenesis³. STAT3, being a pleiotropic protein, activates different gene sets in response to different stimuli like growth

factors, cytokines (such as IL-6 and IL-12), and oncogenes⁴.

STAT3 resides on chromosome 17q21, has structural similarity to other members of the STAT family. It consists of six domains: N-terminal (ND) with a coiled-coil domain (CCD), linker domain, SH2 domain, transcriptional activation domain (TAD), and DNA-binding domain (DBD). The CCD is essential for nuclear localization signals and protein interactions⁵. In resting cells, STAT3 is in an inactive state and is activated via many pathways, such as receptor tyrosine kinases (e.g., PDGFR, EGFR) and non-receptor tyrosine kinases (e.g., Abl, Src)⁶. The most crucial pathway that regulates STAT3 activity is the Janus kinase STAT pathway involving key regulators JAK1, JAK2, and Tyk2. This pathway is activated via extracellular ligand

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interactions with transmembrane receptors, ultimately leading to various transcriptional mechanisms independent of second messengers⁷. STAT3 becomes activated through phosphorylation at a specific tyrosine residue (Tyr 705) in response to the attached stimuli involving the common signal-transducing subunit (gp130), which primes the activation of STAT3 and RAS/MAP kinase pathways⁸. The dimer STAT3 then moves into the nucleus, serving as a transcription activator for various target genes⁹. Usually, STAT3 deactivates within an hour due to the action of various cellular regulators, leading to dephosphorylation and translocation back into the cytoplasm¹⁰.

Nonsteroidal anti-inflammatory drugs (NSAIDs) include

a varied group of drugs that share a common mode of action. They are commonly recommended for fever, inflammation, and pain, and account for a noteworthy percentage of annual drug prescriptions among individuals aged 60 and above^{11,12}. NSAIDs include widely used medications like naproxen, aspirin, paracetamol, indomethacin, mefenamic acid, ibuprofen, diclofenac, and celecoxib. Aspirin is often used in low doses for cardiovascular and cerebrovascular conditions¹³. NSAIDs are antipyretic and analgesic, used worldwide to lessen many pain conditions, from headaches to osteoarthritis. NSAIDs can be administered alone or in conjunction with opioids for severe pain management^{14,15} (Fig. 1).

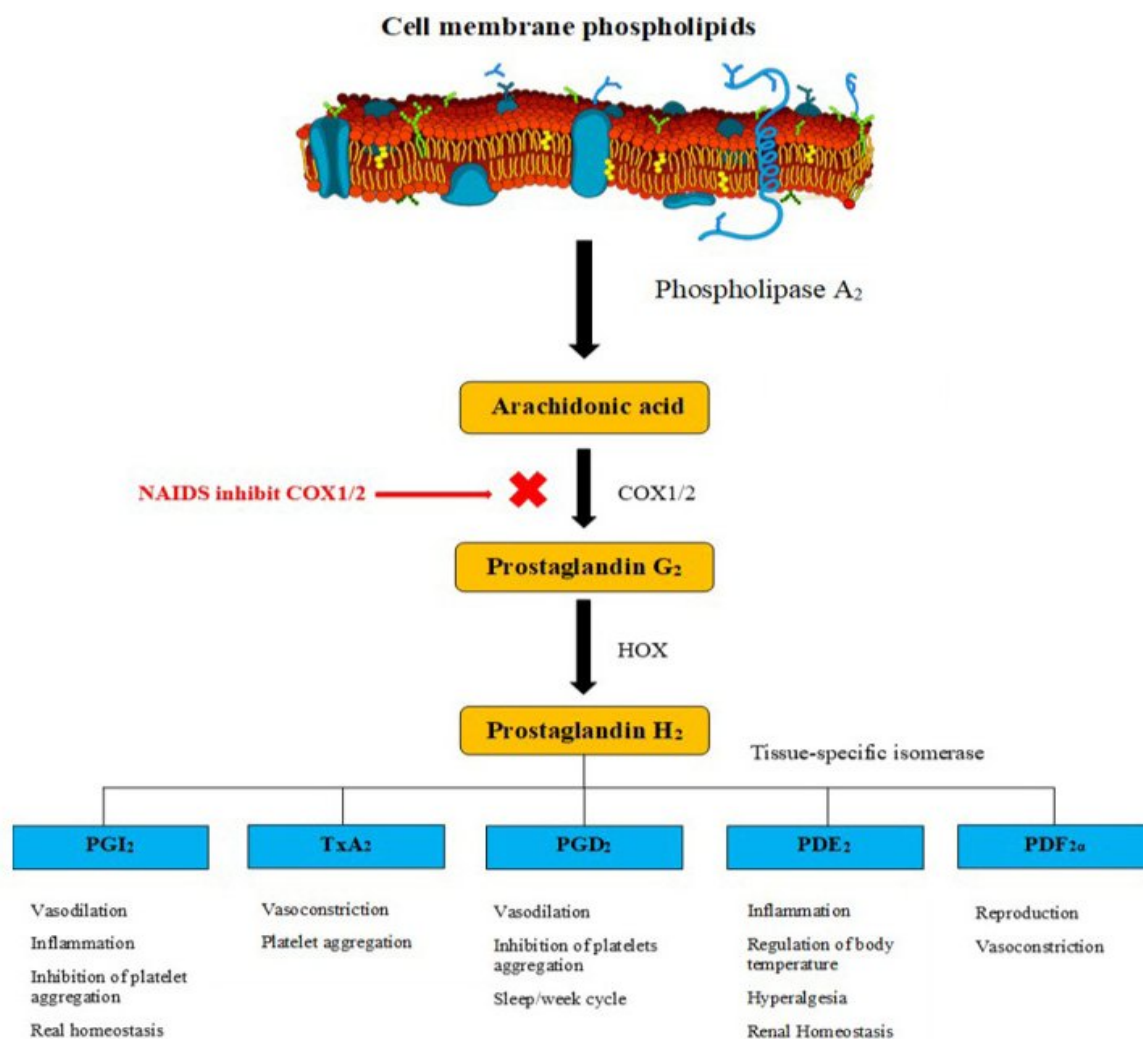


Fig. 1. Mechanism of action of NSAIDs. Arachidonic acid is released from cell membrane phospholipids by phospholipase A₂. It is then converted by cyclooxygenase enzymes (COX-1 and/or COX-2) to the unstable intermediate prostaglandin H₂ (PGH₂) via prostaglandin G₂. PGH₂ serves as the common precursor for tissue-specific prostanooids.

Molecular docking is an advanced technique used to place three-dimensional small ligands at receptor active sites in different orientations. It relies on the concept of induced fit, where successful binding is contingent on the complementary shapes and matching energy profiles of ligands and proteins¹⁶. In this current computational investigation, our main objective is to undertake the repurposing of nonsteroidal anti-inflammatory drugs (NSAIDs) as potential inhibitors of STAT3.

MATERIALS AND METHODS

In this study, a molecular docking tool was used for analyzing the interactions between STAT-3 and FDA-approved NSAIDs. The software tools used included Auto-Dock MGL Tools (<https://ccsb.scripps.edu/mgltools/downloads/>), Auto-Dock vina (<https://ccsb.scripps.edu/mgltools/downloads/>), PyMOL (<https://www.pymol.org/>), Discovery Studio Visualizer 2.5 (<https://discover.3ds.com/discovery-studio-visualizer-download>), and Ligplot+ 4.5.3 (<https://www.ebi.ac.uk/thornton-srv/software/LigPlus/download.html>).

Protein preparation

STAT3 β /DNA complex (PDB code 1BG1) crystalline structure was taken from the Protein Data Bank (PDB) under Research Collaboratory for Structural Bioinformatics (RCSB). Pre-existing ligands and molecules of water were detached, and then polar hydrogens and Kollman charges were added using the tool AutoDock Vina. CASTp server (<http://sts.bioe.uic.edu/>) is a tool to predict the catalytic site, and it was used for STAT-3 protein's binding pockets and cavities predictions¹⁷.

Ligand preparation

Eight FDA-approved three-dimensional structures of studied NSAIDs were taken from the online PubChem database in the format of a structure-data file (SDF). These .sdf format of the ligands were then converted to PDB format using PyMOL and then to PDBQT format using Auto-Dock MGL tools. Discovery Studio Visualizer (DSV) was used to visualize the PDBQT format of these structures.

Docking and visualization

For the grid box and docking parameter file formation, AutoDock Vina was used. Docking analysis viewed all ligand bonds as rotatable and the protein molecule as rigid. A grid box of 40 × 40 × 40 Å was used with center at the coordinates of 115.415, 87.292, and 31.356 for X, Y, and Z. The binding site of 1BG1 was docked with each ligand, and for ligand nine, poses were generated, and the finest pose with the lowest binding energy was taken for further analysis. DSV was used to visualize the docking results,

and for complexes with the lowest binding energy, Ligplot diagrams were generated.

Estimation of drug likeness

SwissADME server (<http://www.swissadme.ch/>) assessed the pharmacokinetics, including absorption, distribution, metabolism, and excretion of ligands¹⁸. With the help of the candidate drug's canonical SMILES drug-likeness were evaluated based on Lipinski's Rule of Five, Egan, Ghose, Muegge's and Veber rules. For further ADMET predictions, the ADMETSar server (<http://lmmd.ecust.edu.cn/admetSar2>) was also used.

Prediction of toxicity

For the prediction of toxicity, the canonical SMILES of NSAIDs were retrieved from PubChem, and then using the pkCSM server (<http://biosig.unimelb.edu.au/pkcsM/prediction>), different levels of toxicity were predicted, such as cytochrome inhibition, skin sensitivity, and hepatotoxicity¹⁹.

RESULTS

In the proliferation of cells, apoptosis, and response of the immune system, STAT3 plays a very important role. The prepared protein and ligand was docked and pharmacokinetics were assessed via online tools (Fig. 2).

The STAT3 (PDB ID: 1BG1) 3D structure contains 296 amino acids. In STAT3, 88 binding pockets are identified by CASTp, being a key target, pocket 2 which contains 22 residues, i.e. MET331, HIS332, PRO333, ASP334, ARG335, ILE467, CYS468, MET470, PRO471, ASN472, TRP474, THR515, VAL563, ASP566, ASN567, ILE569, ASP570, LYS573, LYS574, LYS615, GLU616, LYS642 (Fig. 3). The binding energies, inhibition constants, along binding interactions of all the complexes are given in Table I.

Binding energies and interactions

Both Ibuprofen and Aspirin efficiently bind with STAT3, with -5.6 kcal/mol binding energies and 7.5 μ M inhibition constants. Ibuprofen forms hydrogen bonding with GLU455 and different hydrophobic interactions with LEU378, HIS437, and LEU438. Aspirin forms hydrogen bonds with ARG335, ASP566, and LYS573, along with hydrophobic interactions with ILE467 and PRO471 (Fig. 4).

Naproxen in complex with 1BG1 showed -6.8 kcal/mol binding energy and 3.82 μ M inhibition constant. It forms hydrogen bonding with PHE321, LYS244, and hydrophobic interactions with PRO487 and LEU459 (Table I). Diclofenac, mefenamic acid, and etoricoxib all exhibit -6.8 kcal/mol binding energies and 3.82 μ M inhibition constants (Fig. 4). Diclofenac forms hydrogen bonding with HIS332 and hydrophobic interactions with ILE467 and MET470.

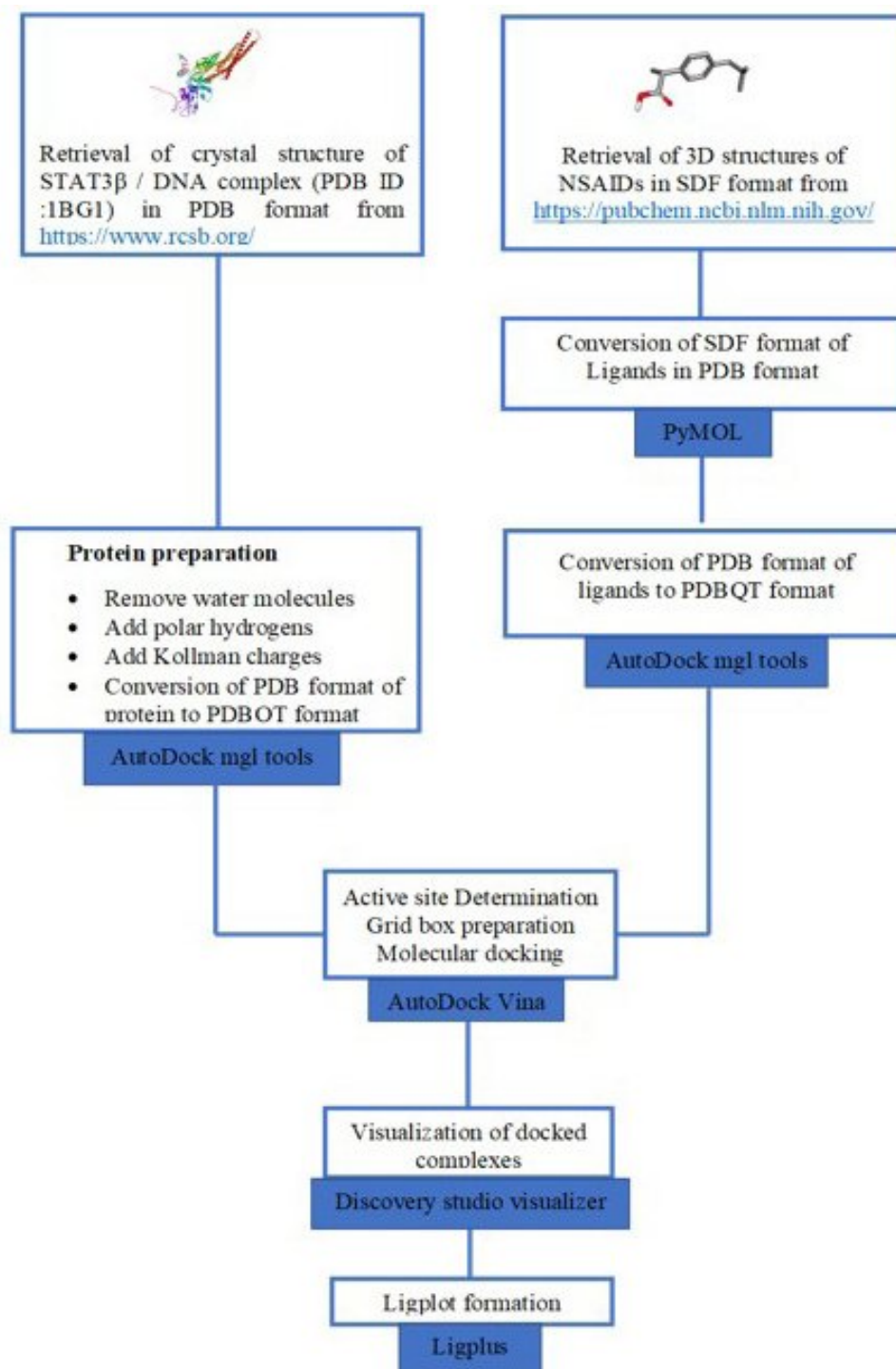


Fig. 2. Summary of docking analysis. Protein (STAT3 β /DNA complex, PDB: 1BG1) and ligand preparation and conversion to PDBQT format was performed using AutoDock tools and PyMOL. Docking performed with AutoDock Vina; complexes visualized in Discovery Studio Visualizer and interactions plotted using LigPlot+.

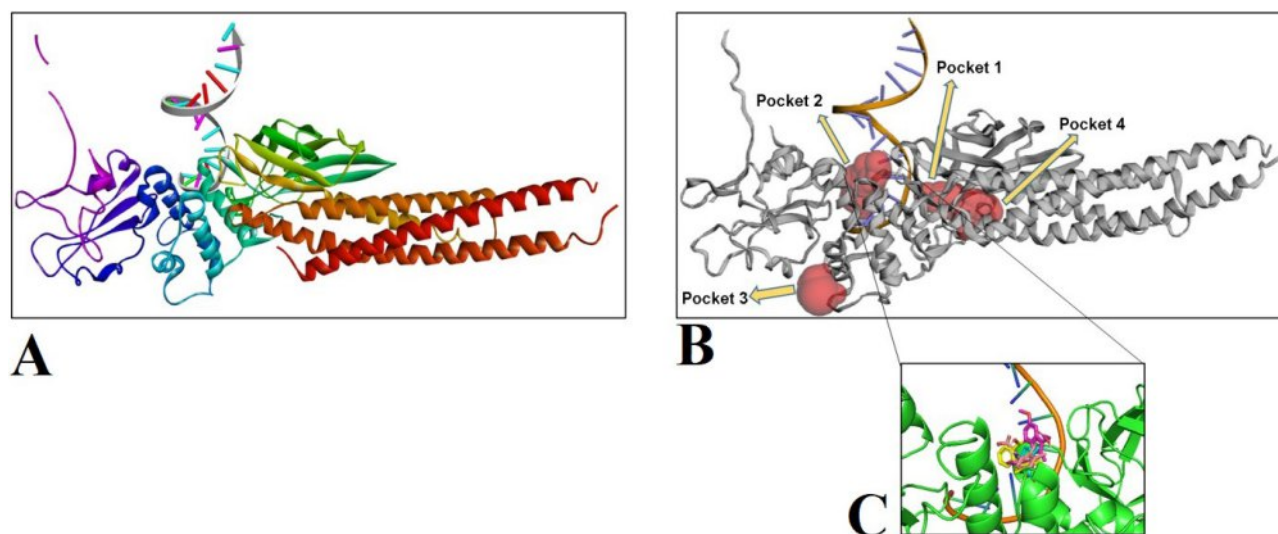


Fig. 3. Structure of STAT3 (1BG1). (A) 3-D geometry obtained using DSV software and online CASTp. (B) It showed the binding cavities and domains within pocket number 1, 2, 3, and 4 are expressed in red color. (C) All the studied ligands showed binding to the binding pocket number 2.

Table 1. NSAIDs in complex with STAT3 showed different hydrogen or hydrophobic interactions as well as halogen bonding visualized via DSV.

NSAIDs	Binding affinity (kcal/mol)	Inhibition constant (μ M)	Interacting amino acid residues (Bond Distance)		
			H-bonds	Hydrophobic interaction (Bond Distance)	Halogen
Ibuprofen	-5.6	7.5	GLU455(2.82Å)	LEU378(3.57Å), LEU438(4.08Å), HIS437(4.56Å)	
Naproxen	-6.8	3.82	PHE321(2.36Å), LYS244(3.50Å)	PRO487(3.97Å), LEU459 (3.97Å), LYS244(4.24Å)	
Diclofenac	-6.8	3.82	HIS332 (3.14Å)	ILE467(4.83Å), MET470(4.55Å), LYS573(3.52Å), ARG335(4.59Å)	
Celecoxib	-7.4	3.57	GLN247 (2.64Å), GLN326 (2.19Å), CYS251 (3.40Å), SER514 (2.73Å), ASP334 (2.02Å), GLY253 (3.28Å)	ILE258(5.12Å), PRO336(5.36Å) PRO333(4.77Å), CYST251(5.10Å), CYS328(5.80Å)	GLU324(3.52Å) GLN247 (2.87Å)
Mefenamic acid	-6.8	3.82	ASP570(1.98Å)	MET470(5.23Å), ILE467(5.15Å), PRO471(5.26Å), ARG335(3.99Å), LYS573(4.70Å)	
Etoricoxib	-6.8	3.82	ASP570(1.96Å)	ASP570 (3.66Å), LYS573(4.44Å), ARG335(3.48Å)	
Indomethacin	-7.5	3.03	ARG335 (2.61Å), ASP334 (2.31Å), LYS573 (2.30Å), HIS332 (2.82Å)	PRO471(4.01Å), HIS332(4.97Å), LYS573 (3.98Å), ASP566(4.09Å), MET470(5.21Å)	
Aspirin	-5.6	7.5	ARG335 (2.15Å), ASP566 (1.92Å), LYS573 (3.56Å)	ILE467(5.00Å), PRO471 (5.46Å), MET470 (4.05Å)	

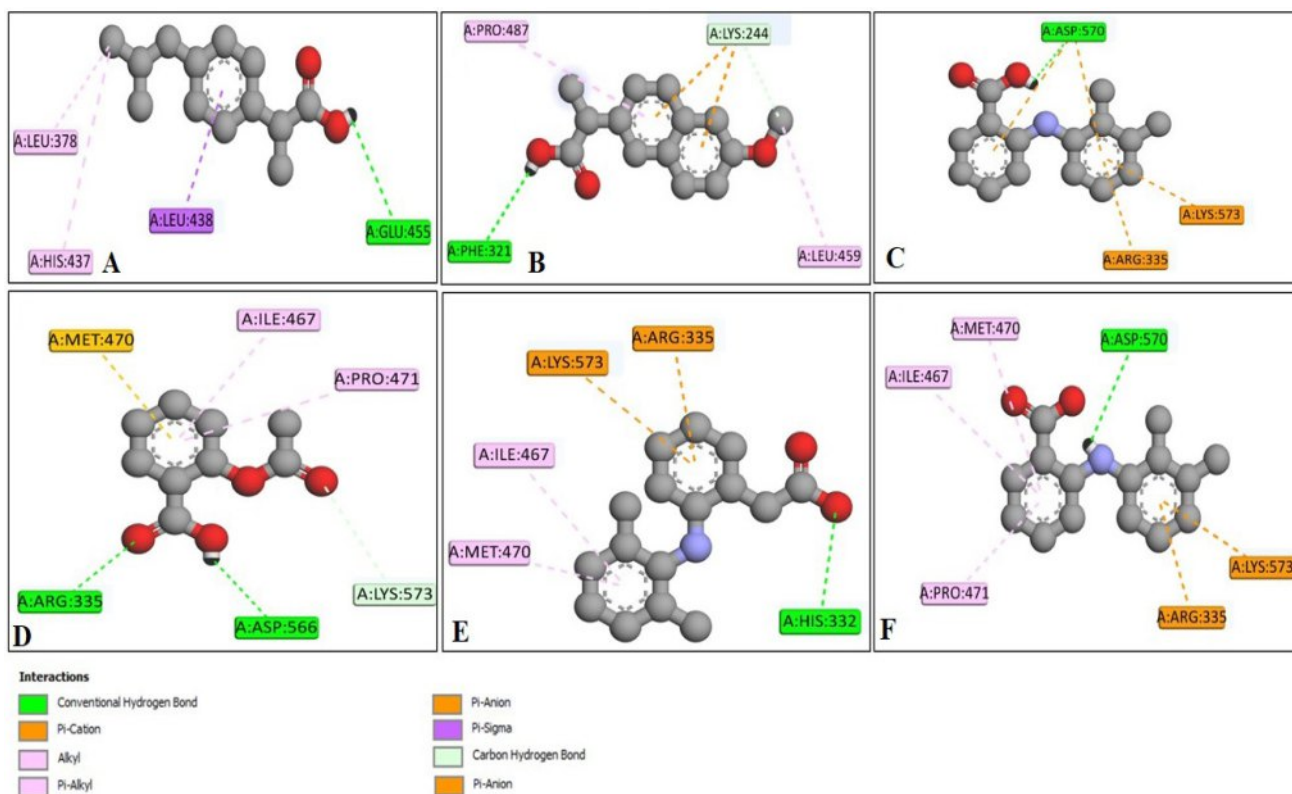


Fig. 4. 2D diagrams visualized via DSV showed binding interactions between 1BG1 and FDA-approved NSAIDs Ibuprofen (A), Naproxen (B), Etoricoxib (C), Aspirin (D), Diclofenac (E), Mefenamic Acid (F). Different types of interactions are shown by different colors as indicated in the Legends.

Table II. Physico-chemical Characteristics of FDA-approved NSAIDs predicted via pkCSM and ADMETsar.

ADMET	Parameters	Coelicoxib	Indomethacin	Etoricoxib
Absorbance	Water solubility (log S) mol/L	-4.45	-3.84	-4.145
	Intestinal absorption (%age absorbed)	92.995	98.649	97.946
	P-glycoprotein I/II inhibitor	Yes	No	No
Distribution	log VDss (L/Kg)	-0.273	-1.633	0.05
Metabolism	CYP2D6 substrate/Inhibitor	No	No	No
	CYP3A4 substrate/Inhibitor	Yes/No	No	Yes
Excretion	Total clearance (Log ml/min/kg)	0.435	0.088	0.174
	Renal OCT2 substrate	No	No	Yes
Toxicity	AMES Toxicity	No	No	No
	Max. tolerable dose (log mg/kg/day)	0.178	1.109	0.064
	Hepatotoxicity	Yes	Yes	Yes

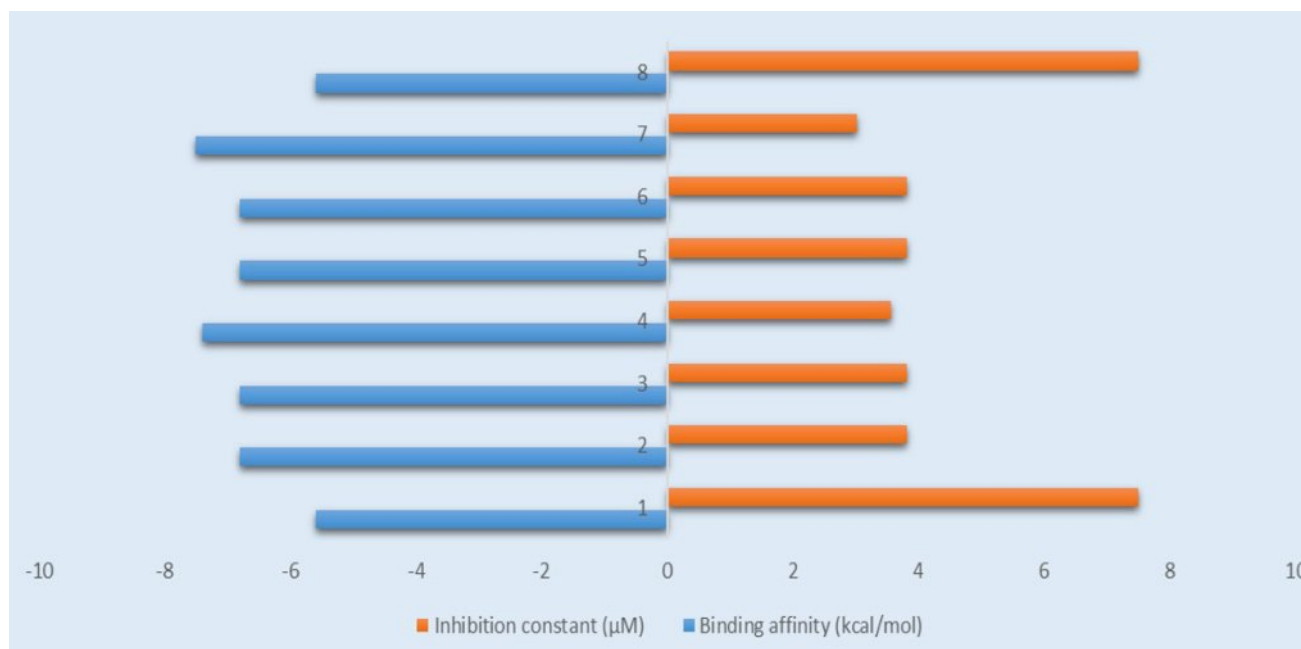


Fig. 5. Graphical Representation of binding energies and predicted inhibition constants (μM) for studied FDA-approved NSAIDs. As indicated clearly, the complex with high binding affinity has the lowest inhibition constant and vice versa.

Table III. Lipinski's Rule of Five for FDA-approved NSAIDs via SwissADME.

Lipinski's rule of five (RO5)	Compounds							
	Ibuprofen	Naproxen	Diclofenac	Celecoxib	Mefenamic acid	Etoricoxib	Indomethacin	Aspirin
Molecular weight < 500kDa	206.28	230.26	296.15	381.37	241.29	358.84	357.79	180.16
Log P (< 5)	3.07	3.04	4.36	5.75	3.75	5.26	3.93	1.31
H- bond donor (5)	1	1	2	1	2	0	1	1
H – bond acceptor (<10)	2	3	2	7	2	4	4	4
Violation	0	0	0	1	0	1	0	0

Mefenamic acid forms hydrogen bonding with ASP570 and different hydrophobic interactions with MET470, ILE467, and PRO471. Etoricoxib forms hydrogen bonding with ASP570 and different hydrophobic interactions with LYS573 and ARG335 (Fig. 5).

Indomethacin exhibits the lowest binding energy, i.e., -7.5 kcal/mol, and inhibition constant of 3.03 μM . It forms hydrogen bonding with ARG335, ASP334, and LYS573, and hydrophobic interactions with PRO471 and HIS332. While Celecoxib shows the second lowest binding energy, i.e., -7.4 kcal/mol and an inhibition constant of 3.57 μM . And forms multiple hydrogen bonds with GLN247, CYS251, and different hydrophobic interactions with ILE258, PRO336,

and PRO333 (Fig. 6).

Drug likeness and ADMET properties

Pharmacokinetics for absorption, distribution, metabolism, and excretion (ADME) are essential to interpret the pharmacodynamics. The different parameters were analyzed via online tools pkCSM and ADMETSar (Table II). Table III. Lipinski's Rule of Five for FDA-approved NSAIDs via SwissADME.

As per Lipinski's rule, the ligands with two or fewer violations have a low toxicity level and have a good absorption value. All the ligands showed no or zero violations, further confirming their bioactive availability (Table III).

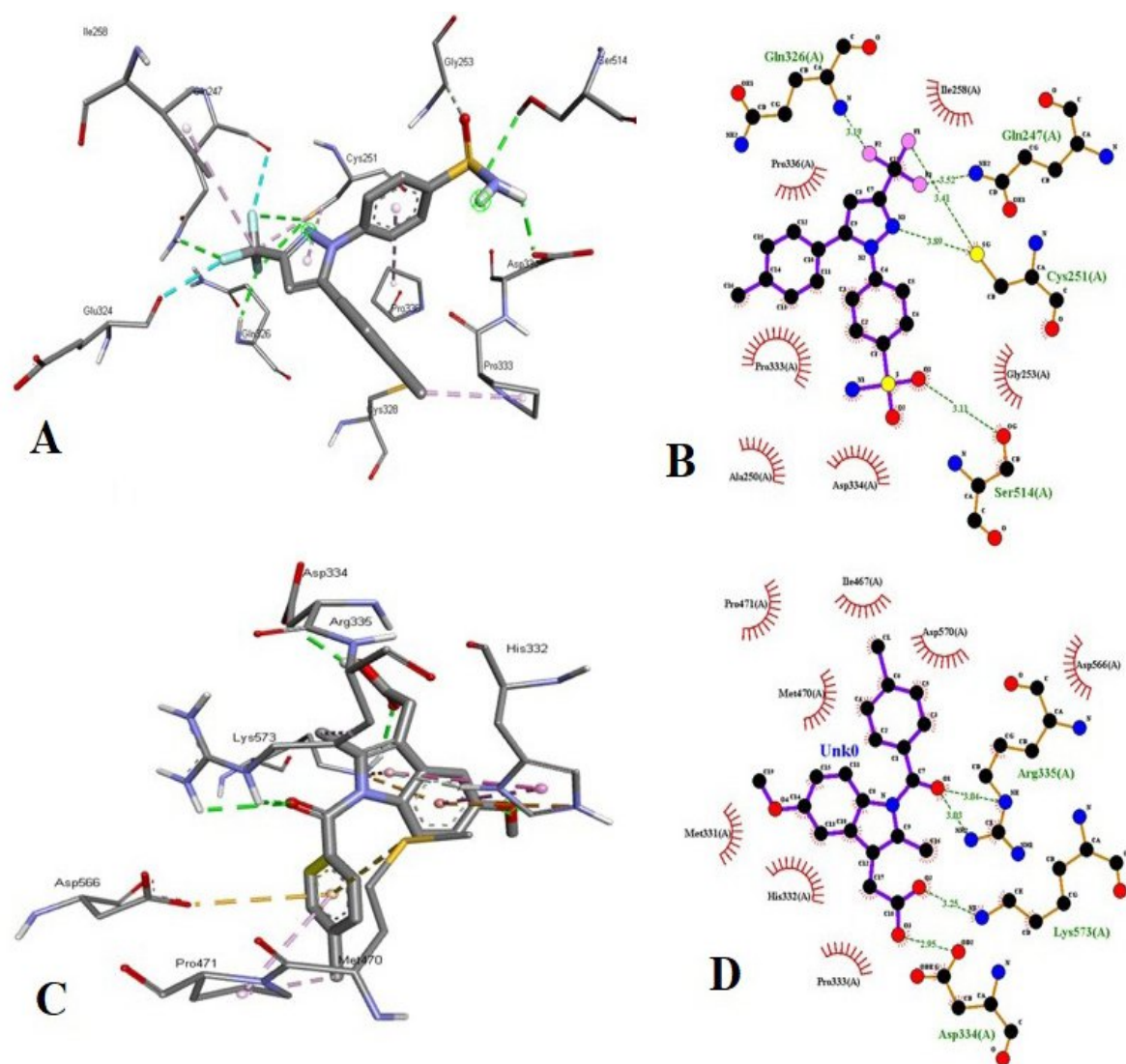


Fig. 6. (A) Three-dimensional (3D) representation of Coelicoxib. (B) Ligplot representation of the complex, green dotted lines show the hydrogen bonds, while the spoked arcs in red show the hydrophobic interactions between Coelicoxib and IBG1. (C) Three-dimensional (3D) representation of Indomethacin. (D) Ligplot representation of the complex, green dotted lines show the hydrogen bonds, while the spoked arcs in red show the hydrophobic interactions between Indomethacin and IBG1.

DISCUSSION

STAT3 is generally known for involvement in the regulation of various important cellular functions like cell proliferation, apoptosis, and the response of the immune system. The continuous activation of STAT3 causes various types of cancers that including breast, liver, colorectal, and pancreatic cancers²⁰. Although STAT3 is an important target of cancer but there is no specific therapy approved by the

FDA that specifically targets its activation, which is a major challenge in the treatment of cancer. As STAT3 plays very important roles in multiple pathways of cells so it's difficult to target it for selective inhibition^{7,8}.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are being recognized as potential anticancer agents. They inhibit tumors through mechanisms such as promoting apoptosis and COX-2 inhibition²¹. This study investigated the potential of eight FDA-approved NSAIDs, Aspirin, Celecoxib,

Diclofenac, Etoricoxib, Ibuprofen, Indomethacin, Mefenamic Acid, and Naproxen, as inhibitors of STAT3 (Table I).

Among these drugs, celecoxib exhibited the highest binding affinity, with -7.4 kcal/mol binding energy and 3.57 μ M inhibition constant. In STAT3, it formed multiple hydrophobic and hydrogen bonds with key residues that indicate celecoxib could be a highly stable inhibitor (Fig. 4). Previous studies have shown that celecoxib is very effective in reducing polyp formation in familial adenomatous polyposis (FAP), which further supports its anticancer properties^{3,22}.

In comparison, ibuprofen exhibited the lowest binding affinity, with -5.6 kcal/mol binding energy and 7.5 μ M inhibition constant. Even though it had weaker interaction with STAT3, the earlier studies show that it might lower the risk of cancer through other mechanisms beyond inhibition of STAT3, especially in colorectal cancer²³.

Other NSAIDs, including diclofenac, etoricoxib, and naproxen, exhibited intermediate binding energies and formed multiple hydrophobic and hydrogen interactions with STAT3. Naproxen, with -6.8 kcal/mol binding energy, created multiple hydrogen bonds, indicating that it is a potential inhibitor of STAT3. Indomethacin also exhibited strong interactions, with -7.5 kcal/mol binding energy, which highlights its applicability in the treatment of cancer. Comprehensively, our docking study supports existing literature, indicating that NSAIDs can be repurposed as potential anticancer agents, specifically focusing on STAT3²³⁻²⁵. Aspirin and ibuprofen are drugs with all the necessary safety profiles that make them candidates for repurposing in cancer treatment. Specifically, aspirin has shown significant potential in reducing the risk of breast, colorectal, and prostate cancers²³. Drug discovery and its feasibility are assessed through toxicity and drug likeness. Evaluating the pharmacokinetic profiling of compounds is essential to ensure their efficiency and safety of use. The ADMET properties of all studied ligands were found to be within acceptable limits, as shown in Table II.

STAT3 β is already in the active or DNA-bound conformation, so the analysis is restricted to only its active state. However, it restricts our ability to explore alternative conformations such as the unbound or inactive state. Because STAT3 proteins undergo substantial conformational changes once bound to DNA, focusing on just the DNA-bound structure may mask important aspects of how STAT3 β modulates interconversion between states, such as activation, dimerization, or interaction with other cofactors.

This computational study provides strong *in silico* demonstrations, but further experimental validation is crucial. Both preclinical and clinical studies are necessary for assessing the safety and efficacy of NSAIDs in cancer patients.

CONCLUSION

This study highlights the promising role of FDA-

approved NSAIDs as potential inhibitors of STAT3, offering a novel approach to cancer treatment. The repurposing of these drugs, with their known safety profiles, could expedite the development of targeted therapies for cancer. Further research on preclinical and clinical evaluations is required to confirm these results in inhibiting STAT3-mediated oncogenesis.

DECLARATIONS

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Authors' contribution

MK conceived and supervised the study. ZA and EF contributed equally to the molecular docking analysis, data interpretation, and wrote the manuscript. AQ contributed to ligand preparation and pharmacokinetic analysis. AJ and MA contributed to data collection and literature review. All authors read and approved the final manuscript.

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Ethics statement

No human participants, identifiable personal data, or live animals were involved. Therefore, institutional ethics approval was not required.

Generative AI and AI-assisted technology statement

The authors declare that they have not used generative AI or AI-assisted technologies in this manuscript.

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The authors have declared no financial or personal conflicts of interest.

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Cloning and Characterization of a Novel *cry2Ad5*-Type Gene from HD29 Strain of *Bacillus thuringiensis* subsp. *galleriae*

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ABSTRACT

Bacillus thuringiensis subsp. *galleriae* HD29 (*Bacillus* Genetic Stock Center ID 4G5; Serotype 5a5b) is known to harbor multiple Cry-type genes such as *cry1Cb*, *cry1Db*, *cry1Ga*, *cry2Ab*, *cry2Ac*, *cry9A* and *cry9B*, which produce crystals consisting of 65- and 130-kDa proteins as major constituents. A new *cry2Ad* gene coding for entomocidal crystal protein is being reported. The main objectives were (i) to clone, sequence and express the new *cry2Ad* gene coding for entomocidal crystal protein of *Bacillus thuringiensis* subsp. *galleriae* HD29 (ii) to determine the toxicity of this new toxin to insect pests and (iii) to determine and characterize the salient features of 3D structure of the protein. Routine methodology was adopted for cloning and sequencing of the gene. For expression analysis, *cry2Ad5* gene was sub-cloned in pET22b expression vector and transferred to *E. coli* BL21 (codon plus) for expression. Proteins expression was analyzed on 12% SDS-PAGE. The expressed protein was used for determining insecticidal activity against lepidopteran (*Helicoverpa armigera*, *Spodoptera litura*) and dipteran (*Aedes aegypti*, *Culex quinquefasciatus*) insects. *cry2Ad5* gene was cloned and sequenced (EMBL accession number AM765844). The protein, listed as Cry2Ad5 (https://www.bpprc-db.org/search_database), was expressed in the form of inclusion bodies, was found to be more toxic to *A. aegypti* than *C. quinquefasciatus* larvae. No significant effect was detected against *Helicoverpa armigera*. The deduced amino acid sequence with 633 amino acid residues shows only 96% identical residues when compared with other δ -endotoxins of the Cry2 family. The predicted 3-D model of Cry2Ad5, when compared with other Cry2Ad type proteins, revealed two semi-conserved and one non-conserved substitution in domain I, and only one semi-conserved substitution was found in the pore forming domain II. The receptor binding motif is present in the loops 1 and 2 of Domain II. A non-significant substitution existed in the interdomain region of the protein. The docking model of Cry2Ad5 was found to be forming 161 and 16 hydrogen bonds with Cadherin and APN of *Helicoverpa armigera*, respectively. The new Cry2Ad5 toxin cloned and expressed from *Bacillus thuringiensis* subsp. *galleriae* strain HD 29 is more toxic to *Aedes aegypti* larvae than to those of *Culex quinquefasciatus*.

KEY WORDS: *cry2Ad5* gene, *Helicoverpa armigera*, Genotyping, *Aedes aegypti* and *Culex quinquefasciatus* entomocidal crystal protein, *Bacillus thuringiensis* subsp. *galleriae* strain HD 29

INTRODUCTION

The HD strains of *Bacillus thuringiensis* harbor multiple *cry*-type genes and produces crystals consisting of 65- and 130-kDa proteins as major constituents. Commercial products containing the HD-1 isolate of *Bacillus thuringiensis* subsp. *kurstaki* have been used successfully to control

certain lepidopterous pests since the early 1970s. At present, bio-insecticides are available for sale, which contain Btk HD-1 expressing Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa proteins or Bt subsp. *aizawai* (Bta) producing Cry1Ab, Cry1C and Cry1D proteins, toxic to *Spodoptera* complex caterpillars including different populations of *Spodoptera frugiperda*, pest on cotton and corn, in the field¹. Much of

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the insecticidal activity of HD-1 is attributed to the three 130-kDa proteins (CryIA) associated with the bipyrimal crystal^{2,3}. However, another class of insecticidal protein(s), P2, also is present in HD-1⁴. The predominant protein that constitutes the P2 cuboidal crystal (now designated Cry2A) has a molecular mass of approximately 65 kDa, and is toxic to both lepidopteran and dipteran insects. Genes encoding Cry2A proteins have been cloned and sequenced from HD-1 and HD-263 of *B. thuringiensis* subsp. *kurstaki*^{5,6,7}. Both genes and their deduced amino acid sequences are identical but share little nucleotide or amino acid homology with other Cry-type proteins^{2,5}. HD37 and HD1 strain also show high pathogenicity against insect pests of irrigated rice⁸.

Alberola et al.⁹ reported natural strains of *B. thuringiensis* which were toxic to larval and adult stages of *Bactrocera oleae*. The PCR analysis of HD12 strain with subfamily primers showed that it harbors *cry1Bb*, *cry1Fb*, *cry1Ha*, *cry1I*, *cry2A* genes, while HD29 strain contained *cry1Cb*, *cry1Db*, *cry1Ga*, *cry2Ab*, *cry2Ac*, *cry9A* and *cry9B* genes¹⁰. These *B. thuringiensis* strains were found toxic to *B. oleae*. The observed toxicity in these strains may be due to these known genes singly, in combination or due to some other unknown genes⁹. Saleem and Shakoori¹¹ also demonstrated that spores of HD29 as well as some recombinant toxins cloned from this strain have shown high levels of toxicity against various lepidopteran as well as dipteran insects. Here we confirm the presence of an unrevealed *cry2Ad* gene in HD29 strain of *B. thuringiensis* subsp. *galleriae*. Zhang et al.¹² have cloned and described the insecticidal activity of *cry2A* gene from *B. thuringiensis*. The main objectives of this study were (i) to clone, sequence and express the new *cry2Ad* gene coding for entomocidal crystal protein of *Bacillus thuringiensis* subsp. *galleriae* HD29 (ii) to determine the toxicity of this new toxin to insects, and (iii) to determine and characterize the salient features of 3D structure of the protein. The present report deals with the cloning, characterization and insecticidal activity of *cry2Ad5* gene from HD29 strain of *B. thuringiensis* subsp. *galleriae*.

MATERIALS AND METHODS

Bacterial isolates

Bacillus thuringiensis subsp. *galleriae* HD29, isolated in Czechoslovakia from *Dendrolimus sibiricus* (Bacillus Genetic Stock Center ID 4G5; Serotype 5a5b) was a generous gift from Professor David J. Ellar. This strain was used to clone novel *cry2Ad5* gene. However, nucleotide sequences of *cry2Ab* and *cry2Ac* in this strain have already been reported by Saleem and Shakoori¹¹.

Cloning and sequencing of *cry2Ad* gene

Total DNA of *Bacillus thuringiensis* subsp. *galleriae* HD29 was isolated according to method described by Kronstad et al.¹³ To amplify full length *cry2Ad* gene from *Bacillus thuringiensis* subsp. *galleriae* HD29, Primer of *cy2Ac* are used as C and N terminal are same in *Cry2Ad* genes.

Cry2AcT F: 5' ATGAATACTGTATTGAATAACGGAAG 3' and Cry2AcT R: 5' CCTTAATAAAGTGGTGAAGATTAG 3'.

The gene was cloned and sequenced using protocols as described earlier^{11,14}. The recombinant T/A clone containing amplified gene was transferred into *E. coli* DH5 α . Recombinant plasmid pTZ/*cry2Ad5* was extracted from recombinant colonies of DH5 α and double restricted with *Nde*I and *Hind*III. Digested fragment of gene was ligated into digested vector pET22b at *Nde*I and *Hind*III restriction site. *E. coli* DH5 α was then transformed with recombinant vectors pET22/*Ad5*. Integrity of recombinant vectors was further confirmed by nucleotide sequencing and restriction analysis.

Expression of *cry2Ad5* gene in *Escherichia coli* (BL21C⁺)

A single recombinant colony containing the gene for the desired protein in BL21 (codon plus) strain of *E. coli* was then incubated in 50 ml LB broth containing ampicillin (100 μ l/ml) and grown at 37 °C overnight with continuous shaking. Then primary culture was seed inoculated (2%) in 100 ml of LB broth (ampicillin 100 μ g/ml) with continuous shaking at 37 °C until OD₆₀₀ was 0.6–0.8. It was then induced with 0.5mM IPTG for different time periods (2, 4, 6, 8 and 10 h). Afterwards, culture was centrifuged at optimum OD₆₀₀ to extract the protein. Expression levels of the protein were analyzed on 12% SDS-PAGE. In all experiments, BL21C⁺ transformed with non-recombinant pET22b plasmid was preceded in parallel as a negative control.

Purification of *Cry2Ad5*

The recombinant *E. coli* cells, expressing the recombinant proteins were pelleted down, resuspended in wash buffer (Triton X-100 1.5%, Tris-Cl (pH 8.0) 50 mM, EDTA (pH 8.0) 10 mM, NaCl 200 mM, DTT 2 mM) and sonicated for 15 min at regular intervals (i.e. 30 seconds sonication and 2 min sonication round), then centrifuged at 9500 rpm (15,971 rcf) for 15 min at 4°C to harvest inclusion bodies. The pellets were then washed with wash buffer till the supernatant turned out to be clear. Afterward, pellet was suspended in 20ml distilled water and stored at -20°C¹¹.

Toxicity bioassays against dipteran and lepidopteron species

To evaluate the specificity of Cry protein, bioassays were performed on different species of Lepidoptera and Diptera. Controls were kept without any Cry protein application to compare the treated and untreated larvae.

Mosquito species i.e. *Aedes aegypti* and *Culex quinquefasciatus* as representative of dipteran insects were used for bioassays. All experiments were carried in three replicas. The 2nd-3rd instar larvae of both mosquito species were suspended in 20ml autoclaved distilled water containing various concentration of *Cry2Ad5* protein in controlled temperature at 25-27°C. Mortality rate were observed after 48 h.

Lepidopteran species i.e. *Helicoverpa armigera* and *Spodoptera litura* were reared on an artificial diet¹⁵ and after

pupation and adult emergence, moths were fed sugar solution. Neonates of these laboratory reared species were used for toxicity bioassays. Various concentration of Cry proteins used for bioassays for diet overlay and diet incorporation method, respectively¹⁴. In diet incorporation method, Cry proteins in the form of partially purified inclusion bodies were dissolved in liquid diet before its solidification.

Cry2Ad gene analysis and clustal X analysis

Sequences of known Cry2A toxins were retrieved. The amino acid sequences of toxins were aligned to see the level of homologies using Clustal W programme from DNA Databank of Japan (<http://www.ddbj.nig.ac.jp/clustalW>). The toxin sequences were aligned using CLUSTAL X ver. 1.83 software¹⁶ and phylogenetic tree was produced.

Secondary structure prediction

The bioinformatics tool “Phyre” was used to predict secondary structure of protein as it provides a simple interface to results¹⁷. The structure was then predicted by giving submitting the FASTA sequence of the query protein in Phyre2 (www.sbg.bio.ic.ac.uk/phyre2). Using Pymol the predicted 3D structure was edited in order to highlight and label the mutations at significant sites i.e domains of the protein.

Protein-receptor docking

In-silico protein docking was performed using ClusPro¹⁸ to analyze spatial and biochemical interaction of amino acids of each Cry2Ad5 protein with two Lepidopteran receptors i.e. Cadherin and APN of *Helicoverpa armigera* and one Dipteran receptor i.e. ALP of *Aedes aegypti*. Following docking, the models were evaluated for the number of bonds and the number of contacting amino acids between proteins and receptor molecules using PDBsum¹⁹.

Statistical analysis

The mortality was determined using Probit analysis.

RESULTS

Cloning and sequencing of the cry2Ad5 gene

Full length cry2Ad5 gene, cloned from *Bt* subsp. *galleriae* HD29 (Supplementary Fig. 1A), was sequenced from both strands and submitted in EMBL accession number AM765844. When compared the sequence of our novel gene from HD29 to all other cry genes simultaneously, it was found indeed a subclass of the cry2Ad gene. The toxin has been listed as novel gene cry2Ad5 (https://www.bpprc-db.org/search_database/). The amplified gene of cry2Ad5 were cloned in pET22b and then transformed into *E. coli* BL21 strain. Restriction analysis confirmed the integrity of recombinant vectors.

Protein production

Recombinant *E. coli* BL21 colonies containing the

recombinant plasmid i.e. pET22/Ad5 was grown in LB broth at 37°C and 120 rpm. The OD₆₀₀ of induced cultures with 0.5mM IPTG at 8 h was found to be optimum and used to harvest inclusion bodies of Cry proteins.

Expression of Cry2Ad5 protein and toxicity bioassays with lepidopteran and dipterans species

SDS-PAGE analysis showed that Cry2Ad5 (65kDa) express clearly (Supplementary Fig. 1B). The toxicity assays of Cry2Ad5 partially purified inclusion bodies were determined by bioassays employing dipteran (*Aedes aegypti* and *Culex quinquefasciatus*) and lepidopteran (*Helicoverpa armigera* and *Spodoptera litura*) insects. Cry2Ad5 proteins were almost equally toxic to the insects with an LC₅₀ value of 1.2 µg/mL for *A. aegypti* and *C. quinquefasciatus* (Fig. 1A). Mortality started after 48 h. There was no significant toxicity data when Cry proteins were applied on the surface of the solidified diet as partially purified inclusion bodies on *H. armigera*, although diet overlay method is more effective than diet incorporation method (Fig. 1B).

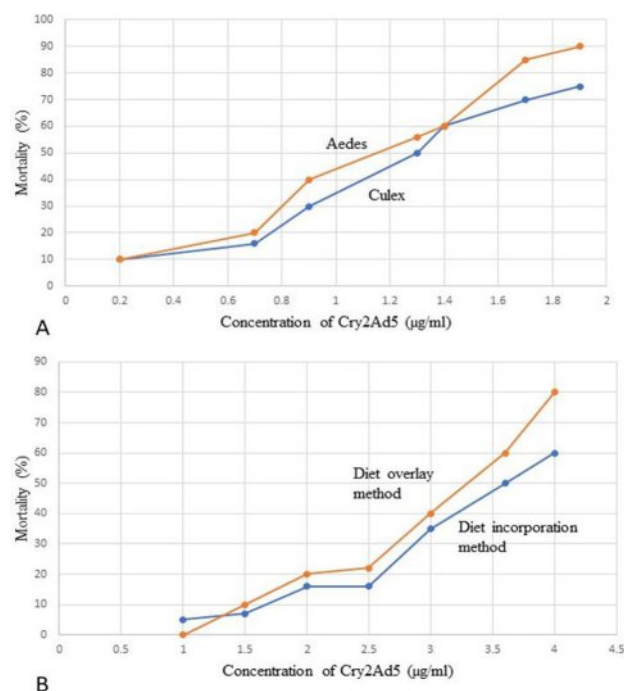


Fig. 1. Effect of different concentrations of Cry2Ad5 protein on mortality of *Aedes aegypti* and *Culex quinquefasciatus* (A) and *Spodoptera litura* (B).

Deduced amino acids sequence and phylogenetic analysis of Cry2Ad

The deduced amino acid sequence of Cry2Ad5 showed 96% of homology with other δ -endotoxins of the Cry2Ad subfamily (Fig. 2A). Figure 2B shows phylogenetic

tree created by CLUSTAL X ver. 1.83 software and TreeView programme. Cry2Ad has two subclusters, one cluster with Cry2Ad1 (Korean origin), Cry2Ad2 (Chinese

origin), Cry2Ad3 and Cry2Ad4 (Pakistani origin) toxins, while other cluster has only Cry2Ad5 toxin. So Cry2Ad5 has least similarity with other toxins of same subfamily.

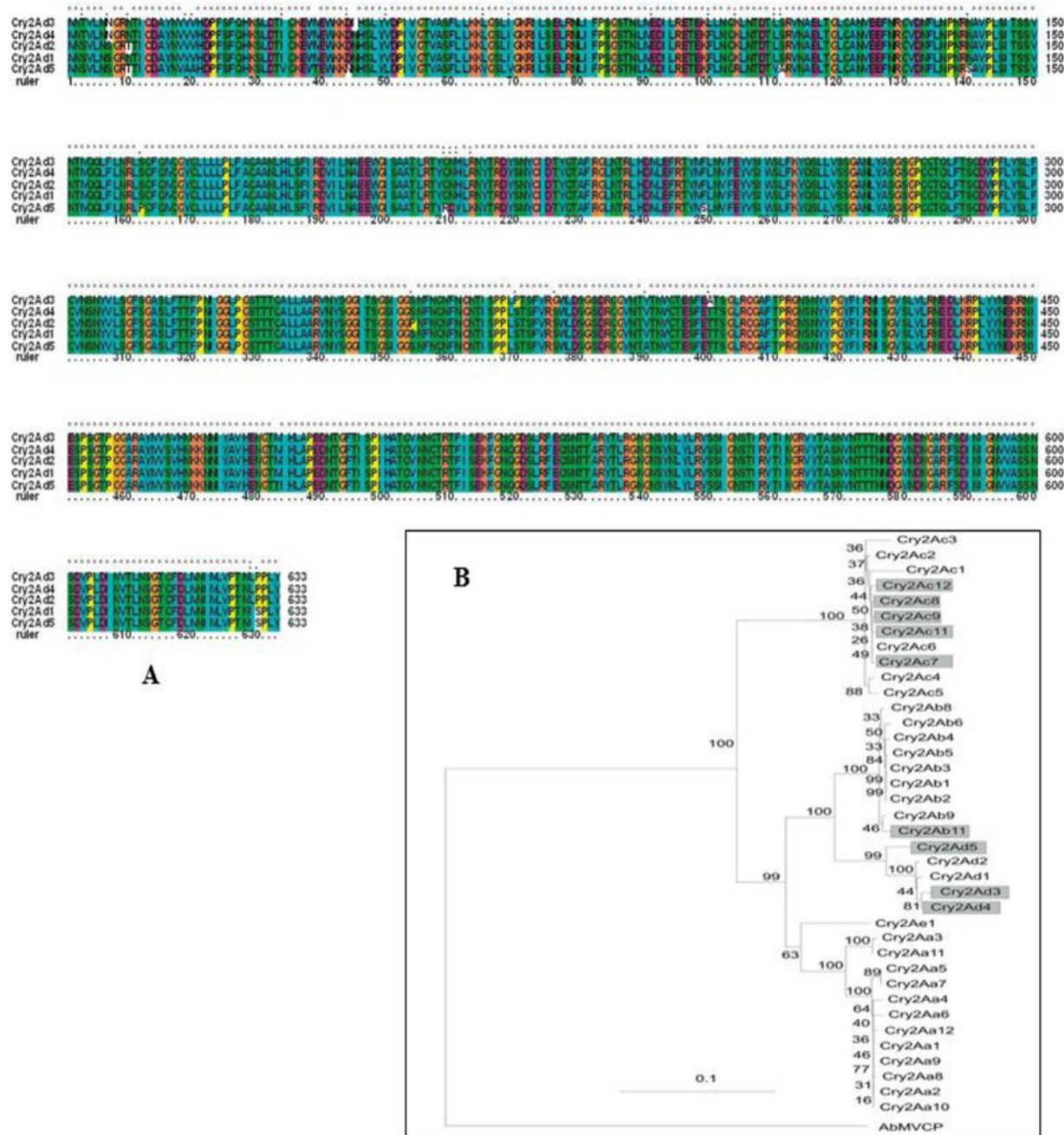


Fig. 2. A) Deduced amino acid sequence homology of Cry2Ad5 with other -endotoxins of the Cry2Ad subfamily. B) Phylogram demonstrating amino acid sequence identity among Cry 2A-type toxins. Toxins reported in the present study (Cry2Ad5) and those from Pakistani isolates are highlighted¹¹. The Tree is rooted against AbMVCP sequence.

Secondary structure prediction

According to “Phyre” Cry2Ad5 has regular local structure of linear segments of polypeptides chains. It contains 33% alpha helix and 25% beta strands while 26% of the region is disordered (Fig. 3). The predicted model of Cry2Ad5 type protein and labeled at substitutions in

comparison with other Cry2Ad type proteins is shown in Figure 4A. Nature of the major substitutions seen in Cry2Ad5 in comparison with other subtypes and level of conservation of a substitution are explained in the Table I. The labeled Cry2Ad5 type protein at substitutions at domain levels was described below:

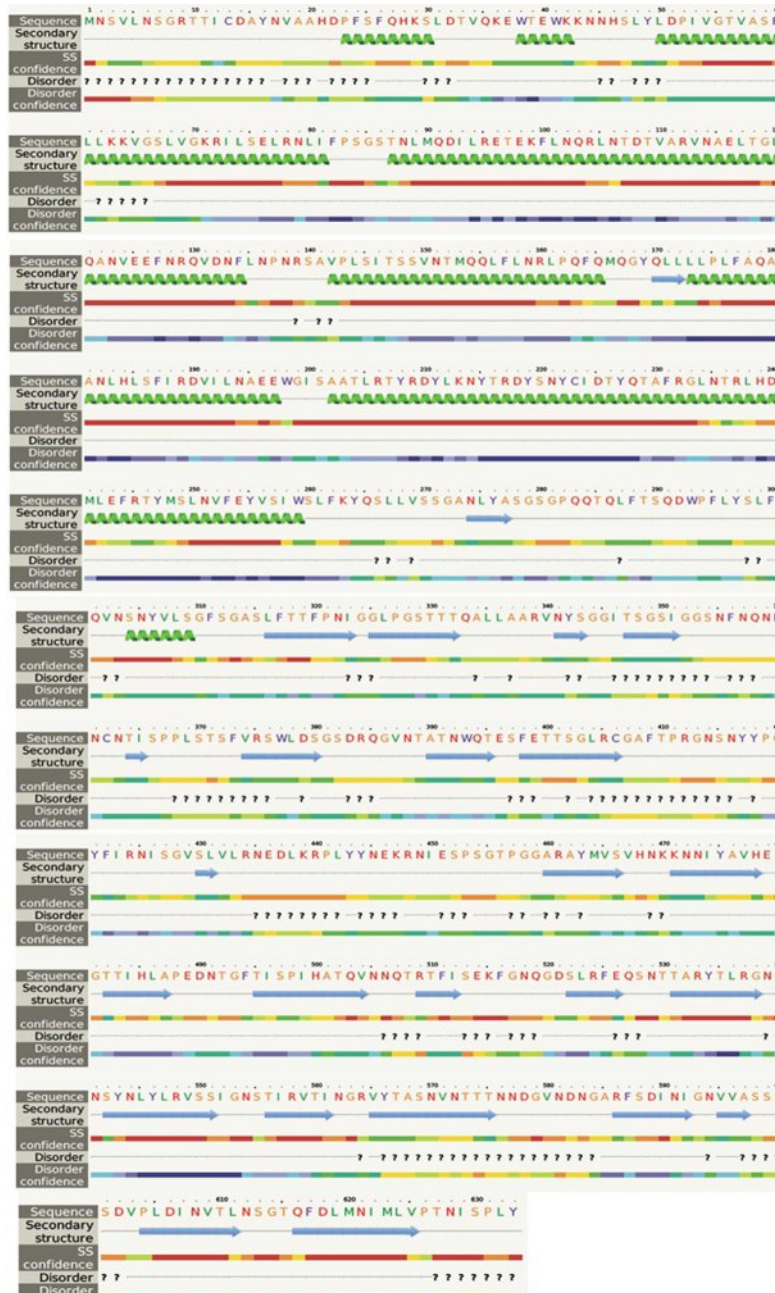


Fig. 3. Deduced secondary structure of Cry2Ad5 using “Phyre”¹⁷.

Table I. Comparison of nature of the major substitutions in Cry2Ad5 in comparison with other subtypes.

Sr. No.	Amino acid position	Cry-2Ad1	Cry-2Ad2	Cry-2Ad3	Cry-2Ad4	Cry-2Ad5	Level of conservation of a substitution	Explanation
1	3	S	S	T	T	S	Conserved	
2	7	S	S	N	N	S	Semi-conserved	
3	10	T	N	N	N	T	Semi-conserved	Asparagine and Threonine both are polar amino acids, hence more or less performs the same function ²³
4	19	V	V	V	V	A	Semi-conserved	Being non-polar and uncritical Alanine does not cause any drastic change by substituting Valine which is hydrophobic in nature and both tends to reside in the backbone structures of the protein but alanine is a smaller amino acid while valine is not hence the substitution is semi-conserved.
5	20	V	V	V	V	A	Semi-conserved	As above
6	34	I	I	I	I	V	Conserved	Ile and Val are closely related amino acids both having aliphatic side chains hence perform the same functions. So the substitution is marked as completely conserved.
7	39	M	M	M	M	T	Not conserved	Methionine has an aliphatic functional group and hence is not that reactive while Threonine contains a free hydroxyl group making it fairly reactive. Hence this substitution can have significant effect in protein's structural conformation.
8	44	D	D	D	D	N	Conserved	Substitution of aspartate by asparagine is a conserved one as both are polar and both are very closely related in their structures.
9	45	N	N	I	N	N	Not conserved	
10	50	V	V	V	V	L	Conserved	Valine and leucine share same aliphatic properties and hence the substitution of a Valine residue by a leucine will not cause any major change in the protein
Domain I- Membrane insertion domain								
11	65	L	L	L	L	V	Conserved	As above
12	69	I	I	I	I	V	Conserved	Isoleucine and valine both tend to reside in hydrophobic cores of the protein so performs the same functions causing the substitution to be conserved
13	91	E	E	E	E	Q	Conserved	Glutamate and glutamine are almost perfect substitutions for each other as both are polar and share very similar properties.
14	100	F	F	L	F	F	Conserved	
15	104	K	K	K	K	R	Conserved	Both arginine and lysine are positively charged residues and can replace each other in a peptide chain, conserving each other's function.
16	110	L	L	L	L	V	Conserved	
17	111	S	S	S	S	A	Conserved	Serine and alanine are similar in the aspect that they both are smaller in size, serine is a relatively indifferent amino acid hence its replacement by alanine does not cause major changes in the protein
18	140	N	N	N	N	S	Semi-conserved	Substitution of Asparagine by any amino acid which is not closely related to it can be significant as lying in a particular domain it can have important functions to perform which cannot be replaced by serine as it is a non-polar and relatively non-reactive when compared with Asn. The substitution is though semi-conserved.

Table continued on next page.....

Sr. No.	Amino acid position	Cry-2Ad1	Cry-2Ad2	Cry-2Ad3	Cry-2Ad4	Cry-2Ad5	Level of conservation of a substitution	Explanation
19	162	S	S	S	S	P	Semi-conserved	Proline's unique properties show that it does not substitute well but it may replace small amino acid as in serine here hence the substitution is labeled as semi-conserved.
20	209	Q	Q	Q	Q	R	Conserved	
21	210	N	N	N	N	D	Conserved	
22	211	H	H	H	H	Y	Conserved	
23	213	R	R	R	R	K	Conserved	
24	249	F	F	F	F	S	not conserved	Phenylalanine has an aromatic side chain and hence is a bigger molecule when compared to a small molecule like serine so the replacement can be significant as phenylalanine might have some roles in substrate binding ²³ and its substitution by serine may affect the protein's function.
Domain II- Receptor bonding domain								
25	354	S	P	S	S	S	Semi-conserved	
26	370	S	S	P	S	S	Semi-conserved	
27	376	S	S	G	S	S	Semi-conserved	
28	390	V	V	V	V	A	Semi-conserved	Being non-polar and uncritical Alanine does not cause any drastic change by substituting Valine which is hydrophobic in nature and both tends to reside in the backbone structures of the protein but alanine is a smaller amino acid while valine is not hence the substitution is semi-conserved ²³
29	400	T	T	A	T	T	conserved	
Inter domain region								
30	482	M	M	M	M	T	Not conserved	Methionine has an aliphatic functional group and hence is not that reactive while Threonine contains a free hydroxyl group making it fairly reactive ²³
Domain III								
31	629	I	L	L	L	I	conserved	
32	630	S	P	P	P	S	Semi-conserved	Proline's unique properties show that it does not substitute well but it may replace small amino acid as in serine here hence the substitution is labeled as semi-conserved.

Domain I: In the first domain comprising of 51-266^h residue²⁰ there are two semi conserved substitutions N140S, S162P (Fig. 4B) and one non-conserved substitution F249S (Fig. 4C). These substitutions can be significant since they lie in the functional domain of the protein. The positions 140 and 162 can be especially crucial as they lie in the region $\alpha 4$ - $\alpha 5$ which are reported to play key role in membrane insertion mechanism²¹.

Domain II: In the pore forming domain (267-472) comprising of 3 β -sheets a semi conserved substitution i.e. V390A can be observed (Fig. 4D). The receptor binding motifs lie in the inter loops of β -sheets i.e. 310-313, 367-379, 438-456 are the residues lying in the loop 1, 2 and 3, respectively²². The detected substitution does not fall in

any of these motifs hence it can be predicted that it might not have any significant effect on the function of the protein. A non-conserved substitution M482T was found in the inter domain region of the protein (Fig. 4E). This may not have any effect on the functional aspects of the protein but can play a role in determining its physical properties as methionine is fairly non-reactive while on the other hand threonine comes under the category of polar and reactive amino acids. But threonine is also known as a fairly indifferent amino acid²³ hence it may not influence any of the properties of protein.

Domain III: No important substitution was found in the third domain i.e. 494-628. But there is a semi conserved substitution in C-terminal extension P630S.

L block (341-412) which determine the dipteran specificity. The non-matching substitutions are shown in magenta color. The changes in the highly conserved block I are highlighted in yellow color (Fig. 5).

Docking of Cry2Ad5 toxins with *Helicoverpa armigera* and *Aedes aegypti* receptors

Using ClusPro online software all the proteins under study were docked with Cadherin (CADR) of *Helicoverpa armigera*. Docking models obtained were then selected on the basis of highest score to analyze them further through PDBsum tool of ExPasy. None of the docked models showed the presence of any salt bridges and disulphide bonds. Cry2Ad5 showed that 9 hydrogen bonds forming amino acid residues predicted docked model with Cadherin of *Helicoverpa armigera* (Supplementary Fig. 2). Moreover, Cry2Ad5 toxin was found to be forming 16 and 31 hydrogen bonds with APN of *Helicoverpa armigera* (Supplementary Fig. 3). Cry2Ad4 toxin was found to be forming 19 and 15 hydrogen bonds with ALP correspondingly with alkaline phosphatase of *Aedes aegypti*.

Non-Conserved Blocks

Cry2Aa: QVNSNYILSGISGTRLNITFPNIGGLPGSTTTHSLNSARVNYSGGVSSG 350

Cry2Ad5:QVNSNYVLSGFSGASLFTTFPNIGGLPGSTTTQALAAARVNYSGGITSGS 350

.....

Cry2Aa **IGATNLNHNFCSTVLPPLSTPFVRSWLDSGTDREGVATSTNWQTESFQT** 400

Cry2Ad5 IGGSNFNQNFNCNTISPPPLSTSFVRSWLDSGSDRQGVNTATNWQTESFET 400

[illegible]

Cry2Aa **TL**SLRCGAFSARGNSNYFPDYFIRNISGVPLVIRNEDLTRPLHYNQIRNI 450

Cry2Ad5 **TSGLRCGAFTPR**GNSNYYPGYFIRNISGVSLVLRNEDLKRPLYNEYKRNI 450

[illegible]

Conserved Block

Cry2A 169 YQLLLLPLFAQAANMHLSFIRDVILNADEW

Cry2Ad5 169 YQLLLPLFAQAANLHLSFIRDVILNAEEW

Fig. 5. Structure-based alignment of Cry2A and Cry2Ad5: A, three non-conserved substitutions in block D (307-340) determines dipteran specificity²⁴ which has been colored yellow while four non-conserved substitutions were found in the L block (341-412) which determines the dipteran specificity and is colored blue. The non-matching substitutions are colored magenta. B, shows the sequence alignment of highly conserved block I of Cry2A and Cry2Ad5.

DISCUSSION

The Cry proteins have been used as bio-pesticide for more than 50 years, and their safety has been demonstrated²⁵.

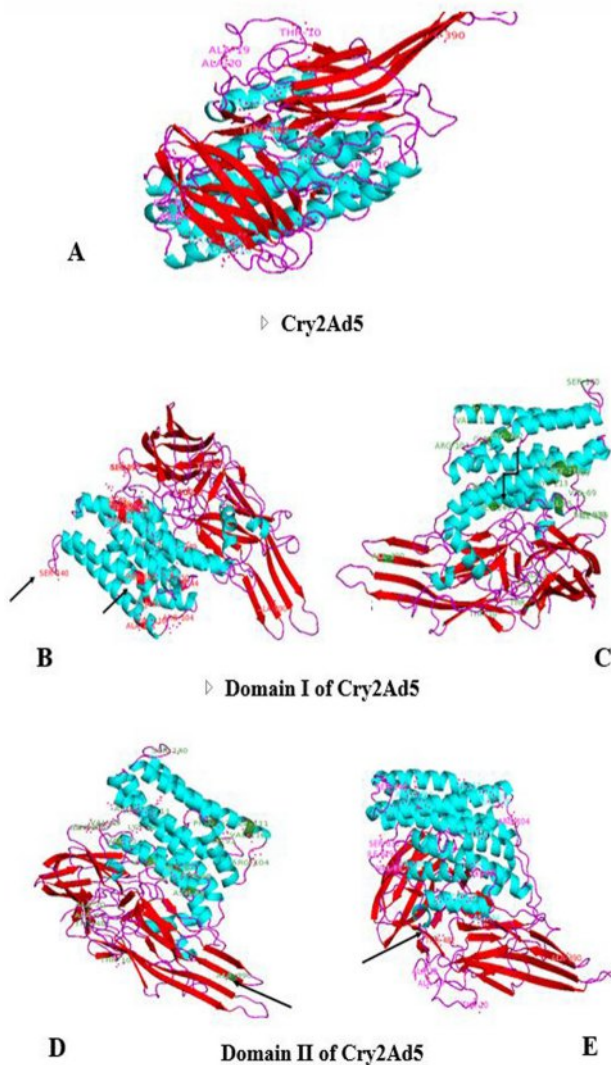


Fig. 4. Predicted models of Cry2Ad5 protein and labeled at substitutions in comparison with other cry2Ad type proteins (predicted through Phyre2 edited through Pymol) (A) showing semi-conserved substitutions N140S and S162P (B) and non-conserved substitution F249S (C) in domain 1, and semi conserved substitution V390A (D) and non-conserved inter-domain substitution M482T (E) in domain II of Cry2Ad5.

Comparison of Cry2Aa protein with Cry2Ad5

Figure 5 shows alignment of cry2Ad5 sequence with the specificity determining regions²⁴. Three non-conserved substitutions in block D (307-340) can be recognized (yellow color) that determine dipteran specificity²⁴ while four non-conserved substitutions shown in blue color are found in the

In this study *Bacillus thuringiensis* subsp. *galleriae* HD29 (Bacillus Genetic Stock Center ID 4G5; Serotype 5a5b) were used to clone novel *cry2Ad* gene. Identification of *cry* gene content by PCR is the most effective technique in screening large native collections when predicting insecticidal activities of individual strains²⁶.

The comparison of novel gene sequence from HD29 to all other *cry2A* genes took place with the help of phylogenetic tree. The amino acid sequence identity among Cry2A-type toxins and toxins reported in the present study (Cry2Ad5) and those from Pakistani isolates¹¹ are highlighted. As it is of great interest to investigate the different *cry2* gene profiles of native *B. thuringiensis* isolates in order to define their distribution, predict their insecticidal activity and detect novel genes or combinations thereof among Pakistani isolates. There are two subclusters of Cry2Ad, one cluster with Cry2Ad1 (Korean origin) and Cry2Ad2 (China origin) toxins, while other cluster has Cry2Ad3 and Cry2Ad4 (Pakistan origin). It is evident from the tree that each of the Cry2 toxins has close amino acid sequence identities and grouped together. While Cry2 toxin isolated from China showed variation and produce two clusters. Cry2Ac2 and Cry2Ac6 formed a separate cluster from Cry2Ac4 and Cry2Ac5. Similarly Cry2Ab3, 4, 5, 6 and Cry2Ab8 produce a different group from Cry2Ab9. *Bacillus thuringiensis* strains are characterized by using a number of different methods to identify their toxicity against different insect orders^{27, 28, 29}. However, information about the distribution of *cry* genes is still limited and does not cover many distinct geographic areas. There is, therefore, the need to search for novel and more potent strains with new pathogenic spectra and wider host ranges, especially in parts of the world that have not been adequately sampled. Pakistan constitutes one such area which needs to be explored.

Protein function is key to any understanding of the consequences of amino acids substitution. According to “Phyre” disordered region is found in Cry2Ad5. These disordered regions have often been found to be involved in protein function and should be taken into account when analyzing predicted functional sites. “Phyre” used a consensus prediction to obtain a confidence value for the predicted secondary structures. Confidence values are displayed from 0 (low confidence) to 9 (high confidence). Two-state disorder prediction score discovered that whether regions of the query are structurally ordered or disordered. The Cry2A type proteins are known to be toxic for lepidopteran and dipteran classes of insect where Cry2Ab and Cry2Ac are specific for lepidopteran class of insects while Cry2Aa is specific for both lepidopteran and dipteran classes. When protein under study is compared with Cry2A by using bioinformatics approaches, it was predicted that Cry2Ad5 type also contained conserved block 1 which is present in all Cry2A proteins^{8, 25}. The two substitutions seem to be conserved as methionine and leucine are closely

related while glutamate and aspartate can also substitute each other well.

The proteins were docked with the receptor proteins of these insects which are involved in the mode of action of these toxins and hence play a vital role in the resistance gaining mechanism against biopesticides³⁰. The docking results of all the models were analyzed and the bonding predicted among the query toxins and receptor proteins contained either hydrogen bonds or non-bonded contact regions among residues. Docking studies on two Lepidopteran receptors *i.e.* Cadherin and APN of *Helicoverpa armigera* and one Dipteran receptor *i.e.* ALP of *Aedes aegypti* helps out to predict experimental proteins had strong tendency to bond to all these receptors³¹.

Saleem and Shakoori¹¹ demonstrated that high toxicity of sporulated *Bacillus thuringiensis* subsp. *galleriae* HD29. Lethal concentrations at 50% (LC₅₀) of HD29 harbor *cry2* gene against *Helicoverpa armigera* (American bollworms) and *Musca domestica* as 62.9 and 479 µg/gm, respectively. It appears that strain HD29 contains a diversity of crystal toxins. Nicholls *et al.*³² isolated a 49-kDa P2 (CryII) crystal toxin from HD29 and showed that it was active against *Pieris brassicae*, *Aedes aegypti*, and *Anopheles gambiae*³². Thus, it appears that strain HD29 contains a diversity of crystal toxins and used as effective bio-pesticides.

CONCLUSION

The amino acid sequence deduced from nucleotide sequence of *cry2Ad5* clone shows 633 amino acid residues having 96% homology with δ endotoxins of Cry2Ad subfamily. The predicted 3-D model of Cry2Ad5, when compared with other Cry2Ad type proteins, show two semi-conserved and one non-conserved substitution in domain I, and one semi-conserved substitution in the pore forming domain II and 19 and 15 hydrogen bonds with ALP of *Aedes aegypti*. The receptor binding motif is present in the loops 1 and 2 of Domain II. A non-significant substitution existed in the interdomain region of the protein. The docking model of Cry2Ad5 was found to be forming 11 and 16 hydrogen bonds with Cadherin and APN of *Helicoverpa armigera* respectively. Potential of *Bacillus thuringiensis* HD29 strain as biological control agent should be investigated against wide variety of insect pests.

DECLARATIONS

Authors' contribution

FS, MSA: Investigation, methodology, validation, writing-original draft. ARS: Conceptualization, investigation, funding acquisition, project administration, resources, supervision, writing-review and editing.

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