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Conclusion:

Considering present findings along with the following discussion, the following conclusions might be derived:

1. Echinococcus granulosus Alkaline Phosphatase serve as a marker of cyst viability.

2. The different characteristic of ALP from hydatid cyst membrane revealed that E. granulosus Alkaline phosphatase is distinct from human isoenzymes more specifically from liver ALP isoenzyme.

Recommandation:

The following, in our opinion should be the focus of future study:

1. The relation between cyst viability and the level of Echinococcus ALP activity.

2. The preparation of specific ELISA test at utilizing the E. granulosus pALP as the antigen.

1.1 Hydatid Disease:

Hydatid disease, Hydatidosis and Echinococcosis are synonyms for one of the oldest recorded diseases in humans dating back to the time of Hippocrates and Galen and was described by Thebesius in the 17th century [1, 2]. The disease is one of the helminthic, particularly Cestodes (Tapeworms), infestation which causes a great deal of morbidity and rarely mortality [3]. It represents a major medical, social as well as economic problem in endemic areas, where high parasite prevalence is found, like the Middle East and Arabic North Africa [4-7].

Hydatid disease is caused by the larvae of cestodes of the genus Echinococcus; at least four species of Echinococcus can infect humans: E. granulosus, E. multilocularis, E. vogeli and E. oligarthrus. Echinococcus granulosus, the most common Echinococcus to cause disease, is seen in the Mediterranean region, Eastern Europe, Africa, South America, the Middle East, Australia, and New Zealand [8-13] . E. multilocularis has a holo-arctic distribution (Alaska, Canada, the entire tundra region) and in some regions of Asia (Russia, China, Japan) and northern Europe (central and eastern France, Switzerland, Austria, Germany) [14]. E. vogeli and E. oligarthrus are endemic in the tropical regions of South America [9, 15, 16].

An estimated 65 million people in endemic areas are infected, this makes Echinococcosis one of the most important zoonotic diseases in the world [8] . Zoonosis or parasitosis is a term used to

depict a parasitic disease in animals that may be accidentally transmitted to humans by ingesting infested raw or undercooked foods, or by drinking contaminated water or fluids, or by skin bites and cutaneous penetration.

This infestation was also described as acyclozoonosis , that is , a parasitic disease that humans share with other vertebrates but which requires at least one other vertebrate (but no invertebrate) host for completion of its life cycle [17,18] (Figure 1.1). Infestation is usually confined to areas where continuous contact exists between humans and certain domestic carnivores such as dogs and some ungulates including cattle, horses, and camels which can act as reservoirs or intermediate hosts [19].

1.1.1 Infestation and The Development of Hydatid Cyst;

The tiny adult tapeworm lives in the intestine of the "primary host", mostly dogs or foxes[20] . The tapeworm releases eggs into the environment with feces, which are ingested by the "intermediate host" of sheep, cattle or pigs. Human, an "accidental host", become infected by ingesting the eggs of the tapeworm, excreted by the final hosts or during normal feeding by a variety of rodents and small lagomorphs[21] . There are two methods of acquiring the disease, either by eating improperly disinfected vegetables or by close contact with dogs.

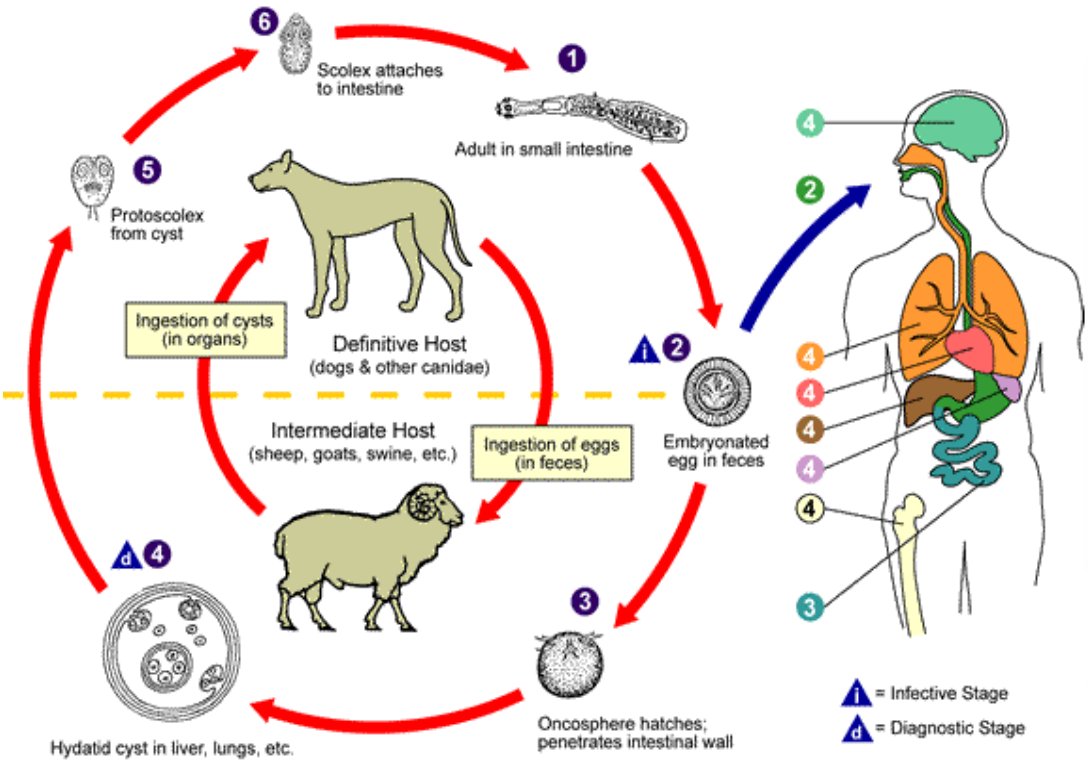


Figure (1.1) life cycle [18]

(1) adult worms in bowels of definitive host. (2) eggs passed in feces, ingested by humans or intermediate host. (3) onchosphere penetrates intestinal wall, carried via blood vessels to lodge in organs. (4) hyatid cysts develop in liver, lungs, brain, heart. (5) protoscolices (hydatid sand) ingested by definitive host. (6) attach to small intestine and grow to adult worm.

Swallowed eggs hatch in the stomach and duodenum. The hatched embryos migrate through the intestinal mucosa into the portal system, mesenteric venules, and lymphatics, to lodge in the liver (60–90%), lungs, kidneys, bones and to any other part of the body [22]. Following implantation of the embryo in the tissue, it attains in about a month the size of 250 microns. Thus it stimulates a conspicuous host tissue reaction where some fibroblasts, giant cells and eosinophils are infiltrated between the endothelial cells that surround the cyst. A second deposit of fibroblasts, with many eosinophils and newly formed blood vessels, will also placed around the cyst. Finally a rigid and resistant layer of fibrous tissue called the "pericyst" laid by the host envelops the cyst [23] . It was suggested that it is found as a result of a precipitin reaction between an unknown factor(s), probably a metabolic product(s) of the germinal membrane or scolices which would act as antigens, and antibodies presumably produced by the host[24] .

Pathologically, hydatid cysts consist of three layers. Besides the pericyst, the hydatid cyst itself has 2 layers of its own which regulate the growth of the cyst, it becomes distinct by the end of the fifth month at a time when it reaches the size of 1 cm. These layers constitute the outer non-nucleated fibrous laminated membrane with nutrient functions, which is also called the "ectocyst", and an inner nucleated DNA-rich and living germinal membrane, known as the "endocyst" [25] .

The endocyst is responsible for controlling permeability and osmoregulation of the cyst wall. This membrane is impermeable to certain products of its own metabolism which

accumulate and maintain an osmotic pressure which exceeds that of the host fluids, thus leading to continual imbibitions of host water [24,26]. According to Schwartz [27],the fluid in the hydatid cyst has a high pressure of water, it is crystal-clear or some time may appear as slightly yellowish fluid [27,28,29] with a specific gravity of 1.005 - 1.009 [29,30] or even higher [31] . It is neutral or slightly alkaline fluid with a pH of 6.7 - 7.2 [31] .

This germinal membrane is also responsible for the development of scolices and the generation of daughter cysts [32]. When hydatid cyst grows larger, some cells of the germinal layer, probably from germinal centers rich in DNA, proliferate toward the cystic cavity and become vacuolated which is then stalked and called brood capsules [25] . From the inner wall of these capsules the scolices developed with their heads invaginated into their own bodies. The brood capsule may remain attached to the wall of the cyst or become detached and float freely in the cystic fluid, known as the daughter cysts. The free scolices and the free brood capsules are collectively called hydatid sand. Daughter cysts development within the mother cyst is an early sign of cyst degeneration, finally the cysts die and frequently calcify gradually [33].The brood capsules and daughter cysts break down, liberating the developed scolices[34]. One cubic centimeter of the hydatid sand contains about 400,000scolices [35] . In fertile cysts, hydatid fluid contain myriads of scolices which are visible as grains of hydatid sand[28].

The scolices have 2 potentialities :first, they can grow into adult worm in 7 to 8 weeks in the small intestine of dogs or other

suitable canines when they are eaten by these final hosts. Second, they can produce secondary hydatid cysts if they are introduced intraperitoneally into laboratory animals ,or if they are spilled during operation, in the peritoneal or pleural cavities of man [36] .

1.1.2 Epidemiology and Types of Hydatid Disease in Human:

In Iraq hydatid disease have been considered as an important public healthy and economic problem [37-42] . It was as early as the period from 1923 through 1949, when 385 cases of hydatid disease in human were reported from Iraq [43] .

Most information on the prevalence and incidence of echinococcosis in human is based on case reports and individual hospital records of surgeries. An estimate incidence in Iraq is between 1/20,000 and 1/50,000 per annum and it is particularly common in rural areas, where most patients had a history of longstanding contact with animals, both domestic (dogs) and farming (sheep) [44].

1.1.2.1 The Distribution of Hydatid Disease in Human;

Although, in most series the liver seems to be the most common organ involved [45,33] . Echinococcosis can affect any organ of the human body from head to toe [45] . Three major types of echinococcosis have been recognized in humans: cystic

echinococcosis (CE), alveolar echinococcosis (AE) and polycystic echinococcosis.

1.1.2.1.1 Cystic Echinococcosis;

Cystic echinococcosis or Unilocular is caused by E. granulosus. Overall, the liver is the most commonly affected organ; however, hydatidosis of thoracic cavity is a very common presentation among adults [45-47] and perhaps the most common among children [48,49] .

Diverse organs in the chest can be affected by the disease either by growing cysts from the liver that traverse the diaphragm or by hematogenous spread [14] . Although the lungs are affected in most cases of thoracic hydatid disease, other thoracic organs can be affected including pleural fissures, parietal pleura, chest wall, mediastinum, diaphragm and heart [50-52] .

Ninety-six cases of human hydatidosis caused by Echinococcus granulosus were diagnosed from surgical records of hospitals in Theqar Province, Southern Iraq during 1989. Many organs such as liver, lungs, peritoneum and spleen were involved, but the liver was most frequently affected. Of 96 patients, 66 (68.8%) showed single organ-involvement and 30 (31.2%) multiple organ-involvement[53].

Epidemiological studies on cystic echinococcosis in Arbil province (Northern Iraq) between 1990 and 1998 showed a high prevalence in dogs, sheep, goats and cattle were 49.5%, 15.0%,

6.2% and 20.6%, respectively.During the same period 99 cases of human cystic echinococcosis (12.4 cases per year) were surgically treated at the two main hospitals in Arbil province, and from this the human occurrence for the province was estimated to be 2 per 100,000 inhabitants [54]. The prevalence of human hydatidosis did not differ significantly over the years, but the study confirmed that hydatidosis is endemic in northern Iraq, and housewives, laborers and farmers appear to be at the greatest risk of infection .

The prevalence of Echinococcus granulosus in stray dogs was investigated in three provinces in Iraq. Of the 150 dogs examined, 57 (38%) were infected with E. granulosus. The prevalence of the worm was higher in the dogs of Theqar province (southern Iraq) (56%) than in those of Al-Tamim province (northern Iraq) (20%) and Diala Province (mid Iraq) (38%) [55]. Moreover, studies in Mosul's abattoir showed 6.57% infection rate in water buffaloes. The infection rate of sheep, cattle and goats were 9.94%, 8.93% and 6.16%, respectively [56].

Recently Shehatha [57], have retrospectively evaluate 763 patients diagnosed with thoracic hydatid cysts and treated surgically at Ibn-Alnafis Teaching Hospital between January 1986 and January 2006. Isolated pulmonary involvement was most common involving 720 patients; these included 660 single cysts confined to one lung, 39 patients with multiple cysts in one lung and 21 involving both lungs. Thirty-two patients had combined hepatic and right lung involvement, six with cysts confined to the chest wall and four confined to the heart. One patient had one cyst in the anterior mediastinum.

1.1.2.1.2 Alveolar Echinococcosis;

Alveolar Echinococcosis, this parasitosis caused by E. multilocularis, is less frequent than E. granulosus, but more aggressive [58] . The infection behaves like a malignant liver tumor, invading and metastasizing to the brain, lungs and other organs [58,59] . It typically appears as a multiloculated cystic lesion or occasionally as an infiltrative tumor like lesion simulating primary or secondary hepatic malignancy or abscesses. If left untreated, virtually 100% of patients will ultimately die within 15 years[20,58,60].

Alveolar echinococcosis is mostly prevalent in Northern Europe and America, but less common in the Middle East where Iran and Turkey are known as endemic areas of E. multilocularis [58]. However, it seems that the disease is found in Iraq as well. Actually, the first documented human case of E. multilocularis in the liver is reported from Iraq. The patient was a 40-year-old female farmer from the north who had never traveled outside her native town of Zakho [61].

1.1.2.1.3 Polycystic Echinococcosis;

Echinococcus vogeli causes polycystic echinococcosis, a relatively recently described echinococcosis endemic in the tropical areas of Central and South America. Humans become infected after a complex cycle by ingestion of fluid/food contaminated with parasite eggs shed in bush dogs' feces. Polycystic echinococcosis shares several similarities with alveolar echinococcosis; the liver is

the target organ and thus the most commonly affected; and it can affect the thoracic organs either by direct extension or hematogenous spread (metastasis) [15,62] By the late 1990s, at least 86 cases had been recognized in the literature [15] , and today around 200 cases have been identified, but are thought to be only the tip of the iceberg [62].

A fourth type of Echinococcus (E. oligarthrus) has been isolated in wild and domestic cats and its larvae are found in agouties and other rodents. The distribution is similar to that of E. vogeli. Fortunately, only a few cases have been documented in humans [15,63-66] .

1.1.2.2 Clinical Presentation and Complications;

Hydatid disease can remain asymptomatic for long periods of time (months to years) or manifest with diverse symptoms depending on the site of involvement [14,45] . The majority are asymptomatic and are discovered accidentally due to certain complications including abscess, parasitic pulmonary embolism, anaphylaxis secondary to cyst rupture, or cyst superinfection, as well as cyst calcifications or a mass on conventional plain abdominal radiographs [67] .

Shehatha[57] have stated that 37% of their patients were asymptomatic with incidental finding of the cysts on routine chest X-ray during medical checkups in military dispensaries or enrolment for new employment, and that symptoms on presentation

depended on location, size and complications associated with the cysts.

1.1.2.2.1 Silent or asymptomatic cyst growing :

Ramsy and Plorde [68] have declared that echinococcosis is usually acquired in childhood , but a latent period of 5 to 20 years take place before diagnosis . In one patient the latent period of a hepatic cyst was as long as 75 years . These findings are of one mind with other investigators who have reported that hydatid disease is typically acquired in childhood and it may , after growing for some years , causing pressure symptoms [29,69] .

Usually the cyst enlarges gradually , and become manifested by its size [29] . Studies on hydatid disease in Lebanon carried out by Frayha[23] suggest that hydatid cyst may grow and survive for 5 to 20 years or even remain the whole life span of its host and get a very large in size . The department of surgery in the American University Hospital in Beirut had reported in 1969 the removal of a multiple daughter cyst from one cavity in the right lobe of the liver containing 22 liters of hydatid fluid .(figure 1.2)

Figure (1.2) Echinococcus organism taken from a hydatid cyst

Moreover, Krupp and associates in 1987 [70] noted that in hepatic lesion , cysts may remain silent for 10 – 20 years or more until it becomes large enough to produce pressure symptoms in the infected organ . similarly , in the case of pulmonary lesions , cyst cause no symptoms, become large enough to obstruct a bronchus , causing segmental collapse , or erode a bronchus and rupture . on the other hand , it was found that brain cyst produced symptoms earlier and may cause seizures or symptoms of increase intracranial pressure [18] .

1.1.2.2.2 Complications :

Complications are the commonest form of presentation of hydatid disease at the time of diagnosis 36 – 40 % of hepatic cysts and 27 -67 % of pulmonary ones have rupture or become secondarily infected [71] , it is the onset of complications that makes the morbidity not much inferior to that of malignant disease [72] .

When a cyst rupture, the escape of hydatid fluid may cause anaphylactic shock or allergic manifestation usually in the form of an urticarial rash and pruritus. If considerable hydatid material suddenly enter the blood stream, serious anaphylactic symptoms or even sudden death may result [73,74] .

Secondary infection of the cyst with bacteria may occur (usually bacteria of gastrointestinal tract). The formation of purulent material results in the death of the parasite and conversion into a pyogenic abscess. In hepatic cyst , suppuration is the second most common complication , caused by bacteria from biliary tract[27] .

Once the parasite dies, this cause the fluid to be absorbed and an encapsuled laminated membrane will be leftovers. In cases of long standing, the walls of the cyst may calcify [29] . In 1989 , Schwartz [27] stated that calcified liver cyst can be diagnosed radiographically as reticulated calcified shadow in the liver . They also found that small calcified cysts in patients with negative serological test needs no treatment. Calcification is more common

in HD of the liver, spleen, and kidney. It can be quite large in compressible organs and can be solitary or multiple [45] .

Ramsey and Plorde [68] and Krupp[70] revealed that cyst calcification does not necessarily mean cyst death . They also found that hepatic cysts of E. granulosus show a smooth rim of calcification in about 50 % of cases. Whereas, pulmonary and brain cysts do not or rarely calcify [45,47] .

Other complications may arises from the pressure effect in the involved organ due to progressive enlargement of the growing cyst [75] . For example , in the liver , pressure of cysts on the biliary ducts or cyst within the bile ducts result in jaundice [72] . In bone , the cysts are semisolid , invade the medullary cavity , and slowly erode bone , producing pathological fracture . Central nervous system involvement may produce epilepsy or blindness [68] .

1.1.3 Management of Hydatid Disease in Human:

The management of cystic echinococcosis depends largely on guidelines established by the World Health Organization (WHO) in 1996 [76] and in 2001 [77,78] , and on local expertise. The main goals of the most appropriate treatment is to ablate the cyst while preserving normal tissue using a minimally invasive therapy that gives the best results with the lowest rates of morbidity and mortality.

1.1.3.1 Diagnosis of Hydatid Disease in Human;

Mass screening in hyperendemic regions using hand-held portable ultrasound scanners and specific serological tests have been applied to detect the early stage of the disease in the asymptomatic population. Some of the imaging features are pathognomonic, allowing a diagnosis of CE in the majority of cases. Computerized tomography (CT) scanning is an accurate test for the diagnosis and classification of the disease. More, and if available, magnetic resonance imaging (MRI) demonstrates cyst contents, size, and multiplanar relations.

Although a variety of serologic tests are available, high false-negative and false positive results have been described [58] . Indirect hemagglutination and Enzyme linked immune sorbent assay (ELISA) tests in association with abdominal ultrasonography can be used as screening tests in high-risk populations [14,79,80] .

However, in a very small number of patients with atypical imaging features of the cysts and negative combined serology tests (indirect hemagglutination and ELISA), a fine needle aspiration biopsy of the cyst may be required for a definitive diagnosis. Definitive diagnosis relies on immunohistochemical and histological analysis [14] . Delay in diagnosis may lead to serious complications or death [74] .

Serology or immunodiagnostic tests are the most useful techniques for the detection of hydatid cysts in human patients [23] . Hydatid fluid is containing the products of the hydatid cyst , thus ,

hydatid fluid antigen obtained from animal or human hosts has been used in the diagnosis of the disease [80] .

Serological tests of hydatid disease varied in their specificity and sensitivity [23,82-84] . The intradermal test (Casoni test) was frequently used since 1911 and its clinical diagnostic procedures was described for hydatid disease patients. Injection of 0.2 ml of sterile hydatid fluid into the skin produces a wheal up to 5 cm in diameter in about 20 minutes in all positive cases , a positive test indicates that patient has or had hydatidosis [85] . A second important test is the precipitin test in which equal amounts of hydatid fluid and patient's serum were mixed , causing different degrees of precipitation according to the extent of the infection . Although the test provides satisfactory results , it is not used any more because it is , at times , difficult to interpret [23] . A more sensitive but less specific test is the Bentonite Flocculation Test , in which an antigen adsorbed on bentonite particles of standard size would flocculate in the presence of specific antiserum [86] . Another modified but more promising flocculation test is the Latex Flocculation Test in which the sensitivity and specificity were improved [87] .

The indirect Hemagglutination Test in which tanned RBC techniques have been successfully employed was applied also to the diagnosis of hydatid disease . Positive results in this test were 88.6% with 0% of false positive, while only 77.2% in complement fixation test and 87.3% in intradermal test with 5.9% and 18.1% of false positive were recorded , respectively [82,84,88,89] .

The Complement Fixation Test in which sterile HCF from man or sheep are used as antigen with serum or cerebrospinal fluid of infected person as source of antibodies. These antibodies provides high percentage (up to 83%) of positive reactions for living cysts in all locations of the body. The complement fixation antibodies disappeared following the surgical removal of the cysts [23] . The sensitivity and specificity of the complement fixation test was improved but is considered not as sensitive and specific as those described for the intradermal and indirect hemagglutination test [82,88] .

In a recently reported Iraqi study, Shehatha[57] , have retrospectively evaluate 763 patients, radiologically diagnosed with thoracic hydatid cysts. The Casoni and the Weinberg complement fixation tests were positive only in 73% and 86% of patients, respectively.

Alternatively, the diagnosis of AE, can be confirmed by ELISA techniques using specific antigens such as E. multilocularis antigen 2 (Em2) [90]. This antigen was initially identified and characterized by Gottstein [91]. The murine monoclonal antibody MAb G11 specifically reacts with an epitope present on the Em2 antigen[92]. This antigen is currently used, in a commercially available, reliable immunodiagnostic test for the specific diagnosis of AE. The Em2 antigen can be specifically detected using the MAbG11 and is not recognized by most of the sera from patients suffering from cystic echinococcosis, caused by the closely related E. granulosus [90,92] .

Later on, Sarciron [93] have studied the immunological properties of the purified alkaline phosphatase (pALP) of echinococcus multilocularis using AE patient sera in ELISA tests. A comparative study was done with echinococcus multilocularis crude antigen (EmC-Ag) and purified ALP antigen (pALP-Ag). When the parasite purified enzyme was used as antigen , the specificity of the ELISA was markedly increased since it reached 100% without any decreased of its sensitivity (100%). The serologic follow –up of AE patients was conducted during several months with these two antigens in three categories of patients: cured, stabilized and aggravated . There was a good correlation between clinical and serologic data when the pALP was used as antigen in ELISA tests. The anti-pALP antibodies titers did change more rapidly than anti-EmC antibodies titers when a recurrence occurred. Modifications of the anti-pALP antibodies levels were also observed during the patient's therapy: (mebendazole, albendazole and Isoprinosine). These results suggest that pALP-Ag should be used for the diagnosis and the follow-up of AE patients[93].

1.1.3.2 Classification of Hydatid Cysts;

An international classification of cystic echinococcosis has been recently proposed by the WHO Informal Working Group on echinococcosis in 2003 (WHO Informal Working Group on Echinococcosis 2003)[94], which is a modified form of the first described and widely used Gharbi's classification [95] , based on ultrasonographic patterns that correlate with the natural evolution and viability of the cyst.

On imaging, a unilocular cystic lesion (type CL) with no visible cyst wall is a nonspecific finding that may represent a simple nonparasitic cyst, or other fluid-filled lesions including a parasitic cyst. Type CE1 corresponds to a type I Gharbi's classification, which describes a unilocular cyst with a visible cyst wall and may contain hydatid sand (snow flake sign). Type CE2 corresponds to a type III Gharbi's classification, consisting of a multivesicular or multiseptated hydatid cyst with internal daughter cysts. Type CE3 corresponds to type II Gharbi's classification, i.e., a cyst with a floating or detached laminated membrane, and to type IV Gharbi's classification, *i.e.*, a cyst with heterogeneous echo-patterns and containing few peripheral daughter cysts. Type CE4 corresponds to a type IV Gharbi's classification consisting of a degenerated solidified cyst that has no fluid component but having a pseudotumoral or tumor-like appearance. Type CE5 corresponds to type V Gharbi's classification, consisting of a cyst with thick reflecting walls.

Cyst viability much depends on the presence of fluid content within the cyst. The five types of cystic echinococcosis classified by the WHO Informal Working Group in 2003[94] were also grouped into three groups: Group 1 (CE1 and CE2) consisting of active and fertile cysts; Group 2 (CE3) consisting of transitional cysts that have started to degenerate, but may still contain viable protoscoleces; and Group 3 (CE4 and CE5) consisting of inactive and infertile cysts. Cysts were also categorized into small (<5 cm), medium-sized (5–10 cm), and large (>10 cm) which can produce a mass effect on adjacent structures.

1.1.3.3 Treatment of Hydatid Disease in Human;

WHO guidelines for hydatid disease state that chemotherapy is the preferred treatment when surgery is not available, or complete removal is not feasible. Management of hydatid disease can be medical, surgical or usually combined. The WHO published an overview of treatment guidelines for hydatid disease in 1996; this stated that surgery was required in patients with impending cyst rupture, haemoptysis, cyst infection, vital organ compromise and extreme pain [76]. There is a tendency toward the application of laparoscopic surgery for uncomplicated superficial cysts larger than 5 cm in size more often than open surgery, simply because of its lower morbidity and mortality rates and the advantage of a shorter hospital stay compared with open surgery. When surgery is contraindicated, chemotherapy with benzimidazoles (albendazole, mebendazole) allows for control of the disease.

Medical treatment using albendazole or mebendazole has been used as primary drug therapy and as an adjunct to surgery to diminish recurrence and potential spread [96]. Todorov et al. looked at factors influencing hydatid response to medical treatment; and found that cyst response had no significant correlation to site but much more to size. The treatment was more efficacious against smaller and younger cysts; also, the presence of daughter cysts should be regarded as an unfavorable factor for treatment response. Cyst multiplicity did not present insurmountable difficulties, provided the cysts were small and a prolonged course of therapy

was undergone. The choice of drug used for the therapy was important, with the results supporting the advantage of albendazole over mebendazole [97].

Horton in 1997[98] reported that albendazole therapy could result in an apparent cure in up to 30% of cases, with a further 40–50% showing objective evidence of response when followed in the short term. Dosage and duration of treatment are of importance, with efficacy of albendazole increasing with exposure up to three months. The current dose of 800mg daily appears to be adequate. Albendazole has been shown to be useful in the management of hydatid disease both when used as sole treatment or as an adjunct to surgery or other therapies [98].

Long-term medical therapy with benzimidazolic drugs used alone has a low cure rate, estimated at 30% [76]. This is why chemotherapy is preferably used as an adjuvant therapy to surgery, which remains the optimal treatment of choice for hydatidosis that has the potential to remove the cyst and lead to a complete cure.

MRI and CT have been used to evaluate response to medical treatment in hepatic disease [99,100] . The use of medical treatment in thoracic disease is controversial; however, it has been attempted with variable success [i.e., a cure rate of up to 30% , persistence of disease in about 50%, and recurrence in up to 20% of all cases] in advanced disease, inoperable cases or patients who do not accept surgical treatment. Improvement is identified as disappearance or decrease in size of cysts as well as a decrease in surrounding inflammatory changes [58,101-105] .

1.2 Alkaline Phosphatases:

Alkaline phosphatase E.C. (3.1.3.1) that catalyze the hydrolysis of a wide variety of phosphate esters at an alkaline pH. They are ubiquitous in nature and are found in many tissues in humans, including bone, intestine, kidney, liver, placenta and white blood cells [106] .

Typical substrates of human alkaline phosphatases include phosphate esters of primary and secondary aliphatic alcohols (*e.g.* β-glycerophosphate), sugar alcohols, cyclic alcohols, phenols, naphthols and nucleoside monophosphates.

Purified alkaline phosphatases from different sources exhibit three types of activities [107] ; which are : hydrolytic, phosphotransferase and pyrophosphatase. Alkaline phosphatase can hydrolyze sulphur-substituted monoesters of phosphorothionic acid, such as cysteamine S-phosphate , this may indicate that two free hydroxyl groups on the phosphate radical are needed for the activity of alkaline phosphatase [108].

1.2.1 Localization and Structure of ALP :

Light and electron microscopic studies demonstrate that mammalian alkaline phosphatase are primarily localized in the absorptive or secretory surface of cells [109,110]. ALPs are found in

the epithelial cells lining the alveoli of the lacting mammary gland [111] ,in the membrane bordering the bile canaliculus[112] , in the outermost surface of the syncytiotrophoblast of the placenta [113] , the small intestinal mucosa [114], in the brush borders of the renal proximal convoluted tubules [115], and sinusoidal surface of the liver[116] .

There have been many histochemical studies of the localization of ALP activity in the central nervous system (CNS). It has been shown that ALP activity occurs in glial cells as well as in neurons and vascular walls [117,118,119] .

Furthermore, at the electron-microscopic level, there have also been reports concerning the localization of ALP in glial cells, and in cells of the CNS blood vessels [120,121], nerve cells, synaptic buttons and myelin sheaths [122]. Further confirmation that this enzyme(s) is (are) connected to cell membranes came from disciplines; first, extraction of lipid membranes with a variety of solvents or detergents such as N-butanol or sodium deoxycholate which disrupts these membranes and solublizies alkaline phosphatase [111] . Second with homogenization and centrifugation, alkaline phosphatase activity is found to be connected with microtonal and cell membrane fractions [123].

Several mammalian and human ALP have been purified to sufficient degree to permit their characterization. All of the ALP studied so far have been found to be a zinc metalloprotein with a serine residue, capable of reversible phosphorylation, located at the active center of the enzyme [124,125] .

The role that zinc plays in the enzyme may be multiple. It is possible that zinc may be of significance to both structure and catalytic activity of the enzyme. Zinc may act to provide the enzyme with one or more of the following: proper conformation, to stabilize this conformation [126], serve as a cofactor in subunit reassociation [127], to participate in substrate binding [128].

The amino acid composition of the purified human placental alkaline phosphatase had been reported by Ghosh and Fishman [129]. It is distinguished by the presence of 50 percent nonpolar amino acids. The complete amino acid sequence of placental alkaline phosphatase, which were 530 residues, and the sequence of amino acids around the serine residue that is phosphorelated has been shown to be Asp-Ser-Gly-Ala . The primary OH-group of the serine is involved in the active site of the enzyme [130] .

Alkaline phosphatase is a glycoprotein possessing terminal sialic acid [129]. ALP of human placenta, bone, liver and kidney contain sialic acid residues but intestinal alkaline phosphatase (IALP) does not [131,132]. Specific glycosylation of each isoenzyme is initiated in the endoplasmic reticulum and completed in the Golgi apparatus [133].

1.2.2 Function of ALP:

The physiological function of mammalian ALP remains unknown [134,135,136]. Their localization to cell surfaces involved in active transport suggested that they may play some role in

facilitating the movement of substances across these cell membranes, but there is no unequivocal evidence of this. There is no reason to believe that the release of phosphate from its esters at alkaline pH is of any physiological importance , and such hydrolyase activity at pH 10 is probably fortuitous, or polarity of the membrane may provide this[106] .

ALP performs no recognizable function in intestinal juice, faeces, milk, urine, bile, serum and lymph. Its presence there most likely results from the shading of membranes rich in alkaline phosphatase into these materials. For example, ALP activity increases in urine following damage to the kidney and rises most sharply in patients entering the diuretic phase of acute tubular necrosis [137]. Similary, ALP in milk is identical to that in the mammary gland epithelium and appears to be bound to membrane fragments in the milk [111,138].

Circumstantial evidence suggests that bone ALP plays some role in the calcification process in bone but its precise function is still not well defined. Its activity in bone appears to correlate roughly with the physiological activity of the bone and with the number of identifiable osteoblasts [139]. The enzyme is probably located within the osteoblasts [139,140]. Alkaline phosphatase isoenzyme of bone had been observed to be elevated in serum in patient with bone disorders such as Paget's disease[141], hyperparathyroidism[142] , rickets[143], and in idiopathic hyperphosphatasia of infancy [144] as well as in normal children[145,146] .

Earlier studies by Madson and Tuba [147] suggested that intestinal alkaline phosphatase performed some function during lipid absorption. They demonstrated that the activity of IALP is increased greatly in both blood and intestinal mucosa in rats placed on a high fat diet. Later studies in man confirmed these findings [148] and showed that the activity of IALP rose sharply in thoracic duct lymph after a fatty meal and later rose in serum [148,149].

1.2.3 Isoenzymes of ALP:

Many enzymes exist in multiple forms. It has been recommended by the IUPAC-IUB commission on biochemical nomenclature (CBN) (1976) that: the term "multiple forms of the enzyme" should be used as a general term covering all proteins catalyzing the same reaction and occurring naturally in the same species. The term isoenzyme should be applied only to those multiple forms of enzymes arising from genetically determined differences in the primary structure and not to those derived by modification of the same primary sequence [107] .

The multiple forms of alkaline phosphatase are not all isoenzymes because the difference between some of them is due to different degrees of sialylation of the same gene product, they are secondary isoenzymes [107] .

Placental and intestinal multiple forms of alkaline phosphatase, are true isoenzymes, they are encoded by separate
Structural genes, bone, liver and kidney multiple forms, are thought to be the product of a single "tissue-unspecific" structural gene

[150,151] . However the "tissue-unspecific" alkaline phosphatases display tissue – specific variations in such properties as electrophoretic mobility [152], and resistance to inactivation by heat [153]. These differences, particularly those between liver and bone alkaline phosphatases, have proved to be of great value in organ-specific clinical diagnosis [154].

It is presumed that these differences can be attributed to tissue-specific differences in glycosylation of a single protein. For example, the charge-dependent properties of liver and bone alkaline phosphatases are dominated by the large and apparently different numbers of sialic acid residues which terminate their respective carbohydrate side chains, and complete digestion with neuraminidase abolishes the difference in anodal electrophoretic mobility between these two phosphatases [150,151].

The Regan isoenzyme and the Nagao isoenzyme occur in various kinds of malignant tumors, the Kasahara isoenzyme is possibly identical with variant alkaline phosphatase, occurs mainly in hepatoma [155,156], although it may be present in carcinoma other than hepatoma. In addition to these isoenzymes another alkaline phosphatase has been reported, which may be a product of tumor tissues, in carcinoma of the pancreas[157] .

Isoenzymes can be measured and distinguished by their electrophoretic mobility, their behavior towards different substrates, activators and inhibitors and their heat stability. Recently , immunoinhibition has been used in the assay of isoenzymes by using antisera to inhibit the activity of one isoenzyme or subunit type . as the relative amounts of isoenzymes of a particular enzyme

vary in different organs, the distribution of plasma isoenzymes found in diseases tends to be similar to that in the organ from which they were released. This helps to identify the affected organ [107]. Table 1.1 shows the most important characteristics of the normal human ALP isoenzymes[107].

Table (1.1): Showing various isoenzyme types of ALP and some of their important characteristics and tissues distribution:

Isoenzymes	pH optimum	Other properties and inhibitors	Tissue present in	References
Placental	10.5	Heat stable, high pH optimum, complete inhibition by L-phenylalanine, 50% inhibited by 0.005 M phosphate, inhibited by L-P-tromotetramisole, inactivated by urea.	Placental tissue	Mclauchlan et al., 1986
Liver	10.0	Inhibited by urea, L-homoarginine, levamisole.	Liver and other tissues in the body.	Fishman, 1974
Bone	9.0	Inhibited by urea, L-homoarginine, levamisole.	Bone, serum and other tissues in the body.	Fishman, 1974
Kidney	9.2	Inhibited by urea, EDTA and levamisole.	Kidney and other tissues in the body.	Fishman, 1974
Intestinal	9.0	Inhibited by L-phenylalanine	Brush border of intestine	Miggiano et al.,1987

1.2.4 Human Serum Alkaline Phosphatases:

The evidence available suggest that in normal individuals the serum alkaline phosphatase is derived from four sources , liver, bone, intestine and in placenta. There is no evidence available to suggest that other tissues may contribute [106] .

The heterogeneity of nonspecific human serum ALP has been demonstrated by electrophoresis, chromatography, heat stability, immunologic means, and by using organ-specific inhibitors. Three types of phosphatases have been observed in normal human serum. However, Boyer [158] reported that at least 16 bands of nonspecific ALP activity were evident in human sera following electrophoresis on hydrolyzed starch gel, although not all of these bands appear in the same individual.

The alkaline phosphatase of normal serum in adults appears to be mainly derived from the liver with a small, variable intestinal component. The relatively small amounts and the diffuse nature of the bone band on electrophoresis led to the believe that the bone isoenzyme is virtually absent [107] .

In inactivation studies, however, suggest that substantial part of the normal adult ALP is a phosphatase of presumed bone origin. The contribution from bone is normally greater in infancy childhood and adolescence [107] .

1.2.4.1 Elevation of the serum alkaline phosphatase:

Elevation of the serum ALP occurs in a wide variety of clinical situations. The highest levels are found in patients with bone disorders associated with increased osteoblastic activity and individuals with disorder affecting the liver may cause a moderate increase in the serum ALP [106] . In the great majority of patients with an elevated serum ALP, the elevation is due to an increase in either the bone or liver isoenzyme in serum [146] .

Any obstruction of biliary system (e.g., stone, carcinoma, primary biliary cirrhosis); increased serum ALP is a sensitive indicator of intrahepatic or extrahepatic cholestasis. Whenever the alkaline phosphatase (ALP) is elevated, a simultaneous elevation of 5′-nucleotidase (5′-N) establishes biliary disease as the cause of the elevated ALP. If the 5′-N is not increased, the cause of the elevated ALP must be found elsewhere (*e.g.*, bone disease)[159], in patients with alcoholic cirrhosis where the increase is due to the intestinal fraction [160], and in patients with certain malignancies, particularly lung carcinoma , Regan isoenzyme [161] . Diseases of the intestine almost never associated with an increase in the IALP isoenzyme in serum [162] . Kidney ALP is also not ordinarily seen in serum, although this has been reported in patients during the rejection of renal homografts [106] . The exceptions are found in pregnancy where the increase is due to the placental isoenzyme [163].

Moderate elevations of ALP are seen in disorders not primarily affecting the liver or bone, including : myeloid

metaplasia, congestive heart failure, intraabdominal infections [146] , stage I or II Hodgkin's disease [164], and inflammatory bowel disease [165] . In these disorders , the elevated ALP appears to be of hepatic origin and often associated with inflammation of the portal triads of the liver.

1.2.5 Physical properties of ALP isoenzymes:

Alkaline phosphatase of various tissues differs in one or more of its physical properties, so this advantage has been taken in order to identify its tissue origins [133] .

Heat inactivation was first employed in this way to identify bone ALP by its heat sensitivity. Later , urea inactivation was found to make much the same distinctions. Next , electrophoresis has become popular , employing different supporting media such as starch gel , polyacrylamide gel and cellulose acetate membranes . Refinements such as isoelectric focusing in polyacrylamide gel have been the last to be developed . Chromatographic separations based on molecular weight and charge have been successfully used also to distinguished alkaline phosphatases isoenzymes from each other [133] .

1.2.5.1 Heat inactivation:

Heat sensitivity can be used to distinguish between bone, liver, intestinal and placental alkaline phosphatases [166] . The ease of measurement, in this case , has contributed to make this one of

the most commonly used tools in ALP isoenzyme analysis of biologic fluids.

In the original identification of the Regan isoenzyme , and in the characterization of Nagao isoenzyme , the heat stability of these placental- like enzymes was of critical importance in their characterization [134] .

1.2.5.2 Urea inactivation :

A denaturation of kidney alkaline phosphatase by urea was demonstrated by Butterworth and Moss in 1966 [167] . selective denaturation of tissue phosphatases occurs in the order kidney > liver > bone > intestine > placenta [168] . Bahr and Wilkinson demonstrated under their conditions a 50 percent activation , in some case of liver alkaline phosphatase by 0.5M urea.

1.2.5.3 Electrophoretic migration:

Electrophoresis is the name given to the movement of charged particles through an electrolyte subjected to an electric field. The rate of migration of particles of similar charges will depend on the number of charges each carries .

Several electrophoretic techniques have been used to separate and distinguish the isoenzymes of ALP . Although electrophoresis cannot be used as the sole indicator of the origin of a raised ALP activity , only electrophoresis will reveal the presence of an abnormal band . Currently available electrophoretic media do

not completely separate the two most clinically relevant isoenzymes, bone and liver [107] .

The sharpness of separation depending upon the extent to which each fraction is homogeneous in its mobility [169]. The electrophoretic mobility of the isoenzymes is affected by the medium used . liver alkaline phosphatase normally runs as an alpha-2 band , bone runs in the alpha-2 / beta region as does placental ALP. Intestinal isoenzyme is usually found in the beta-2/gamma region [107] .

Acrylamide gel has the advantage that it is easier to prepare and is more inert , the pore size can be varied in a controlled manner and over a wider range [169] . Polyacrylamide gel electrophoresis is the preferred technique as the separation of bone and liver bands is clearer , particularly on vertical slab gels [107] .

1.2.5.4 Optimum pH:

The optimum pH of ALP is affected by the type and concentration of the substrate and buffer, and the presence of activators or inhibitors. Different pH optimums have been observed for ALP from different organs [133] .

Although in vitro studies show that the pH optimum of this enzyme range from 8.2 to 10.7 , it is generally believed that this enzyme is also active in its natural environment at physiologic pH.

At low β-glycerophosphate substrate concentration, such as may occur intracellularly, the pH optimum of intestinal ALP is

7.35 [170] . Histochemical study also showed that ALP is active at pH 5 [171,172,173] .

1.2.5.5 inhibition by organ –specific inhibitors:

Organ-specific inhibitors are inhibitors that preferentially inhibit alkaline phosphatase of a specific organ. These include the inhibition of human and rat IALP by L-phenylalanine [174] , human placental ALP by L-tryptophan [175] and L-leucine [176], and human bone and liver alkaline phosphatases by L-homoarginine [133] , by imidazole[177] and by levamisole [178] .

All these inhibitions are stereospecific (only the L-isomers of the amino acid are active inhibitors) and are noncompetitive [134]

The inhibitory effect of Zn^{2+} appears to be exerted independently of the tissue of origin of the enzyme [179] .

Complete inhibition of the ALP activated by Mg^{2+} was observed after the addition of at least an equimolar quantity of EDTA to an assay mixture [179] . Phosphate , borate,oxalate and cyanide ions are inhibitors of all forms of the enzyme . glycine inhibits apparently by complexing the activating Mg^{2+} [135,180] .

1.2.6 Echinococcus Alkaline Phosphatases:

In the search for parasite viability markers and chemotherapeutic targets, alkaline phosphatase as a membrane-bound metabolic enzyme, is a good candidate. Despite decades of research, its physiological role is still controversial. Although possibly involved in transport processes[181,182], its importance in

protein phosphorylation/ dephosphorylation has been hypothesized [183] and denied [184] .

Preliminary investigations have focused mainly on ALP in hydatid fluid. A study conducted by Devi [185] on 44 different cyst fluids (cow, sheep , ox) revealed that ALP was present in both fertile and sterile cysts, and no significant difference was found between ALP levels in the three hosts. The authors also observed that ALP activity in the sera of these hosts were significantly higher than its activity in the respective HCF. These findings are in concord with those of Aziz [186] who found lower ALP activity in HCF as compared to human serum.

Frayha and Haddad in working with sheep hydatid cysts , revealed that ALP activity in the fresh protoscolices , was significantly higher than its activity in the cysts fluid. They also found that ALP was not detected in the HCF collected from secondary cysts of experimentally infected albino mice[187] .

Conventionally, investigations on echinococcosis have primarily involved animal experimentation. The alkaline phosphatases from larval stages have been purified and characterized in E. multilocularis metacestodes [188] and in the E. granulosus (sheep strain) hydatid membranes [189] .

E. multilocularis possess a high alkaline phosphatase activity. Purification and biochemical characterization of the E. multilocularis alkaline phosphatase provided evidence that this glycoprotein seems to be an important enzyme for the parasite, since its activity was reported to be 50 times greater than hepatic

tissue ALP of control and infected animals [188] . The distinct differences from the corresponding host hepatic enzyme involved in the purification of EmALP, as well as its thermostability, have led to the conclusion that the parasite and the host hepatic enzymes were intrinsically different molecules [188,190] . Afterward, it was shown that it may be used to reflect parasite viability and to monitor the course of infection and therapy of the disease [93] .

The parasite enzyme was found to exhibit original properties compared to the corresponding enzyme from sheep liver tissue: a several-fold increased activity, resistance toward heat denaturation, differences in response to various ALP inhibitors, and slight differences in apparent molecular weight and in isoelectric point.

The biochemical characterization of the purified EmALP [188,190] revealed that the apparent molecular weight of the enzyme monomer and its isoelectric point matched those originally reported for the Em2 antigen[91] . Using in vitro-generated E.multilocularis metacestodes, it was shown by immunofluorescence and immunoelectron microscopical analysis that the Em2 antigen was a surface-bound molecule, primarily expressed in the laminated layer adjacent to the germinal layer of the metacestodes [191].

The fact that both Em2 and EmALP are predominantly localized on surface-associated structures, and thus on the regions of the parasite which interacts with the host, clearly indicates that these two antigens may play important roles in the host–parasite relationship. It is conceivable that the phosphatase activity expressed on the parasite surface may represent an important factor

with respect to the host immune defense, especially with regard to host immune cell recognition. A functional relationship between these two antigens can be postulated and future studies will focus on elucidating the functional significance of both EmALP and the Em2 antigen in terms of host–parasite interactions. A logical hypothesis would be that EmALP and Em2 are closely related in situ, as well as metabolically, and that Em2 antigen could derive from EmALP [93] .

The Echinococcus ALPs thus differ from the mammalian liver-type ALPs by their isoelectric point [188,189] and their resistance to heat denaturation; the 3 enzymes of sheep liver, E. granulosus and the E. multilocularis can be further distinguished with selected inhibitors and finally, the apparent molecular weight and amphiphilic character discriminate between the E. granulosus and the E. multilocularis ALP, which therefore appears as the most original molecule. Preliminary experiments showed that both enzymes are recognized immunologically by patients [192] and that their biochemical interest as chemotherapeutic targets should be enhanced by their immunological importance as markers of parasite viability.

The thermal stability of the Echinococcus ALPs is remarkable[193], and recently Miura [194] reported that selective removal of the O-linked sugar moieties and sialic acid linkage alters the sensitivity to heat exposure of liver- and bone-type ALPs. Thermoresistance is also a characteristic of the mammalian placental ALPs [195] , and has also been described in a shrimp ALP [196] and a fruit fly ALP [197] .

Inhibitors which differentiate between the placental, intestinal and bone/liver/kidney alkaline phosphatases [195] showed that the Echinococcus ALPs cannot be grouped with any of these 3 types. In addition, L-homoarginine, levamisole and $ZnCl_2$ can be useful to discriminate between the 2 parasite enzymes and the liver-type ALP [190] .

More recently, alkaline phosphatase from hydatid cyst fluid (HCF) was purified and characterized by Vatankhah for comparison between fertile and sterile HCF. Samples were obtained from slaughtered sheep and then sterile and fertile Echinococcus granulosus cysts were separated. ALP was purified from aspirated cyst fluid and biochemical parameters were determined. Sera from patients with hydatid disease and patients with other parasitic diseases including fascioliasis, taeniasis and also sera from uninfected controls, were collected and used in immunoblotting experiments with ALP from sterile and fertile HCF as antigen. The authors showed that ALP activity in fertile HCF was significantly more than in sterile HCF. There were also some differences between the kinetic parameters and biochemical characteristics of ALP in fertile and sterile HCF. Moreover, Immunoreactive bands were clearly observed when sera from hydatid infected patients were tested with ALP from fertile HCF as the antigen. However, this method revealed no cross-reaction between purified ALP from sterile HCF and sheep liver tissue. These findings suggest that there is some variation in the immunochemical characteristic of ALP from fertile and sterile HCF[198].

1.3 Study Aim and Protocol:

Delcacho have stressed that one of the reasons why E. granulosus cysts are described as a public health problem is the lack of effective chemotherapy[199].

Reviewing the literature bears in mind the possibility of an effectual and successful chemotherapeutic treatment in the early stages of cyst development, as indicated by many reported studies [96,97,98]. Thus, the major problem in the management of HD seems to be related to the lack of an efficient routine diagnostic test capable of identifying asymptomatic patients at early stages of the disease. Consequently, the need for a consistent diagnostic test, applicable for mass screening in hyperendemic regions, has become mandatory.

Much attention has been focused on the multilocularis species which represent the major type affecting western countries[58]. These cumulative efforts have recently evolved a commercially available and reliable immunodiagnostic test for the specific diagnosis of AE. Unfortunately, the test fails to identify most of the patients suffering from cystic echinococcosis, caused by the closely related E. granulosus[93].

Hence, this will detract from the valuablity of the test in districts, where E. granulosus is well known as the main endemic

type, including Iraq, and necessitating the requirement for further studies with regard to E. granulosus.

Following the lead by Vatankhah [198], the present study was conducted to :-

1. Estimate the kinetic parameters and biochemical characteristics of ALP in fertile and sterile cyst membranes from patients with hepatic cystic echinococcosis.
2. Comparisons were aimed to reveal whether these two cyst membranes enzymes are different from each other and from the Human liver- type ALP.
3. Kinetic and biochemical properties of the ALP isolated the affinity of these enzymes to their substrate, as well as the effect of other factors including reaction pH and temperature. Heat inactivation and the effect of specific inhibitor, namely L-phenylalanine, are also to be examined on the activity of the three ALP enzymes.

3.1 ALP activity in hydatid cyst membrane:

Results for ALP activity in hydatid cyst homogenate (HCH) is presented in table 3.1. Different activities were observed in patients with Group 1 CE1 (9.1 and 10.3 K.A.U for patient 1 and patient 2, respectively). No activities were noticed in homogenates from patients with Group 3 CE4 or CE5, ALP activity still not able to be detected even when larger volume of the HCH sample were used for the estimation.

Based on the WHO classification, cysts from patients 1 and 2 are believed to be active and fertile cysts, while cysts from patients 3,4,5 and 6 are grouped as inactive and sterile cysts[94]. Considering the study of Frayha and Haddad who stated that ALP activity in the fresh protoscoleces was significantly higher than its activity in the cysts fluid and that ALP was not detected in the HCF collected from secondary cysts of experimentally infected albino mice[187]. Accordingly, present results may reflect the viability of the cyst and can be explain by the presence or absence of fresh protoscoleces in fertile or sterile hydatid cyst, respectively.

Failure to detect any ALP activity in group 3 CE 4 and CE 5 hydatid cyst in the present study, along with a higher activity in group 1 CE 1 cysts, may suggest E. granulosus alkaline phosphatase as a marker of cyst viability, and may explain the similarity between sterile E. granulosus Hydatid cyst fluid and liver host enzyme observed by Vatankhah as compared to fertile E. granulosus hydatid cyst fluid. However, it should be stressed here that cyst viability was not examined by ours. Further study

Table (3.1) Alkaline phosphatase activity in HCH.

Patient	Cyst type	ALP activity in HCH (K.A.U)			Mean
		patch1	patch2	patch3	
1	Group 1 CE1	6.5	12.2	8.6	9.1
2	Group 1 CE1	13.2	7.6	10.0	10.3
3	Group 3 CE4	ND	ND	ND	ND
4	Group 3 CE4	ND	ND	ND	ND
5	Group 3 CE4	ND	ND	ND	ND
6	Group 3 CE5	ND	ND	ND	ND

ND : Not detected

relating cyst viability to the level of echinococcus ALP activity is needed to provide support to this notion.

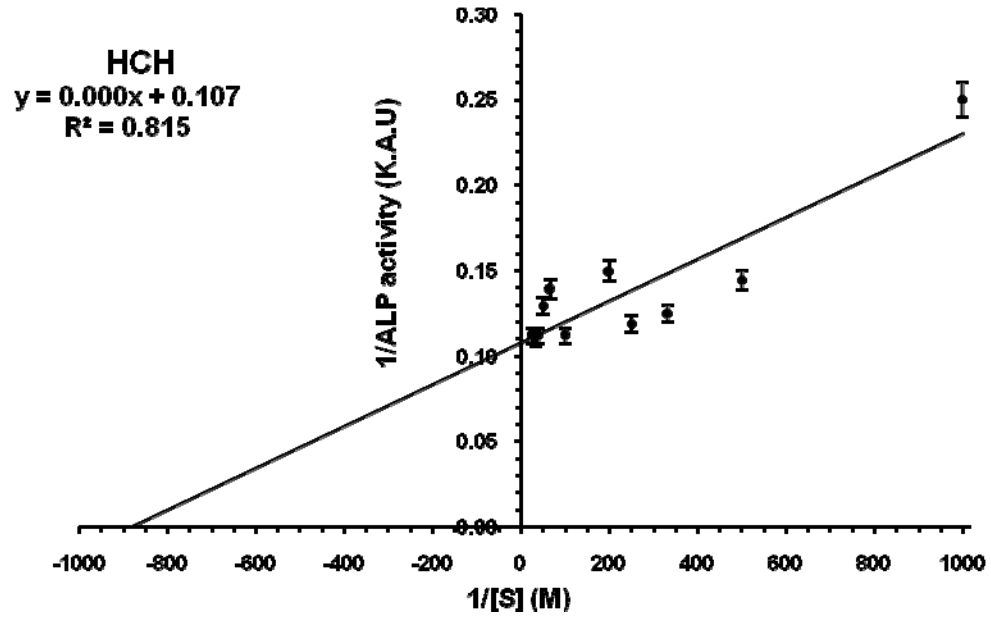
Moreover, different activities were obtained from different patches of the same cyst (6.5, 12.2 and 8.6 K.A.U for patient 1; 13.2, 7.6 and 10.0 K.A.U for patient 2). Since ALP is a membrane bound enzyme, sonication of the HCH before centrifugation, is necessary to ensure the complete extraction of the enzyme from hydatid cyst membrane [93], such facility is not available in present study. However, as all the patches were subjected to the same procedural setting, this could not justify the wide variability seen between the different patches in the present study. The irregular distribution of an active germinal layer in the same cyst, on the other hand, may account for the interpatches differences observed in our study.

3.2 Biochemical Characterization of ALP enzyme:

3.2.1 Affinity to Substrate:

The relation between ALP activity and substrate concentration in HCH and serum were present in figure 3.1. Lineweaver-Burk plot was adopted to estimate K_m values. The estimation of V_{max} , on the other hand, should be carried out on purified enzyme , the crud extract prepared in our study can hardly be utilized for such estimation.

Six sets for serum and four HCH sets were carried out. Alkaline phosphatase from HCH shows a higher affinity for disodium phenyl phosphate as indicated by a lower K_m (table 3.2), compared to that of serum ALP (0.928 and 2.037 mM for HCH and serum, respectively). Similar results were observed by Vatankhah who reported a higher affinity of ALP from fertile echinococcus HCF as to the sterile HCF and host liver enzyme[198]. High affinity of E.multilocularis ALP to p-nitrophenyl phosphate was also described by Sarciron et al.[188]. This may suggest that Echinococcus granulosus ALP differ from that of human liver-type.



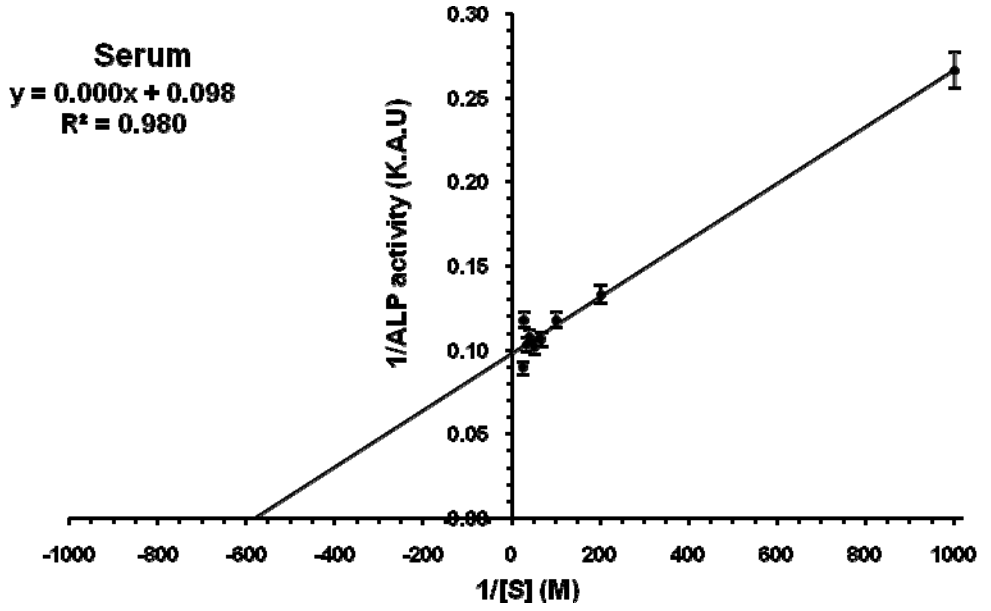


Figure (3.1) Lineweaver-Burk for HCH alkaline phosphatase (Result from 4 separate experiments) and Serum ALP (from 6 separate experiments)

Table (3.2) Affinity of serum & HCH Alkaline phosphatase to disodium phenyl phosphate

Sample	Serum	HCH
number of sets	*6*	*4*
Equation	*y = 0.0002x + 0.0982*	*y = 0.0001x + 0.1078*
R2	*0.9801*	*0.8152*
Km (mM)	*2.037*	*0.928*

3.2.2 Optimum pH:

Six sets for serum (figure 3.2) and 4 sets for HCH (figure 3.3) were performed to explore the effect of pH on ALP activity. It is apparent there is two values of optimum pH, the measured value was shown in figure 3.4, optimum pH for serum ALP 10.78, while the apparent value from the data obtain implies that the pH from HCH is around 9.5 and as compaired with serum around 11.2 these finding could be of variable biological importance and need to be clearified further. Finally, optimum pH for serum ALP was higher than that for HCH (10.78 and 9.9 for serum and HCH, respectively calculated from the diagram). Similar figures were reported by Vatankhah for echinococcus fertile HCF [198], though, Lawton et al. have cited a pH optimum of 9.0 for the parasite ALP [190].This inconsistency can be attributed to the use of different substrate or substrate concentrations in the experimental approaches [202] .

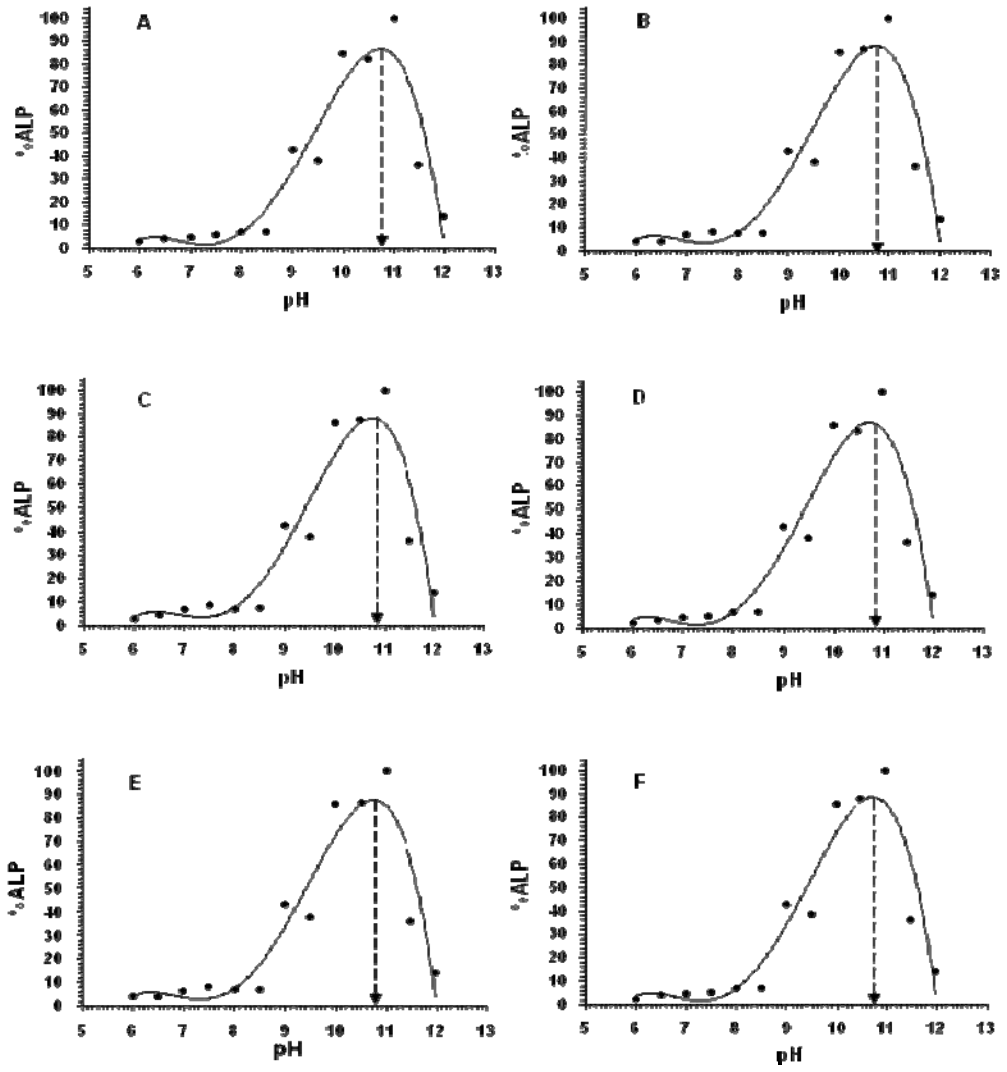


Figure (3.2) pH optimum on serum ALP (6 separate experiments)

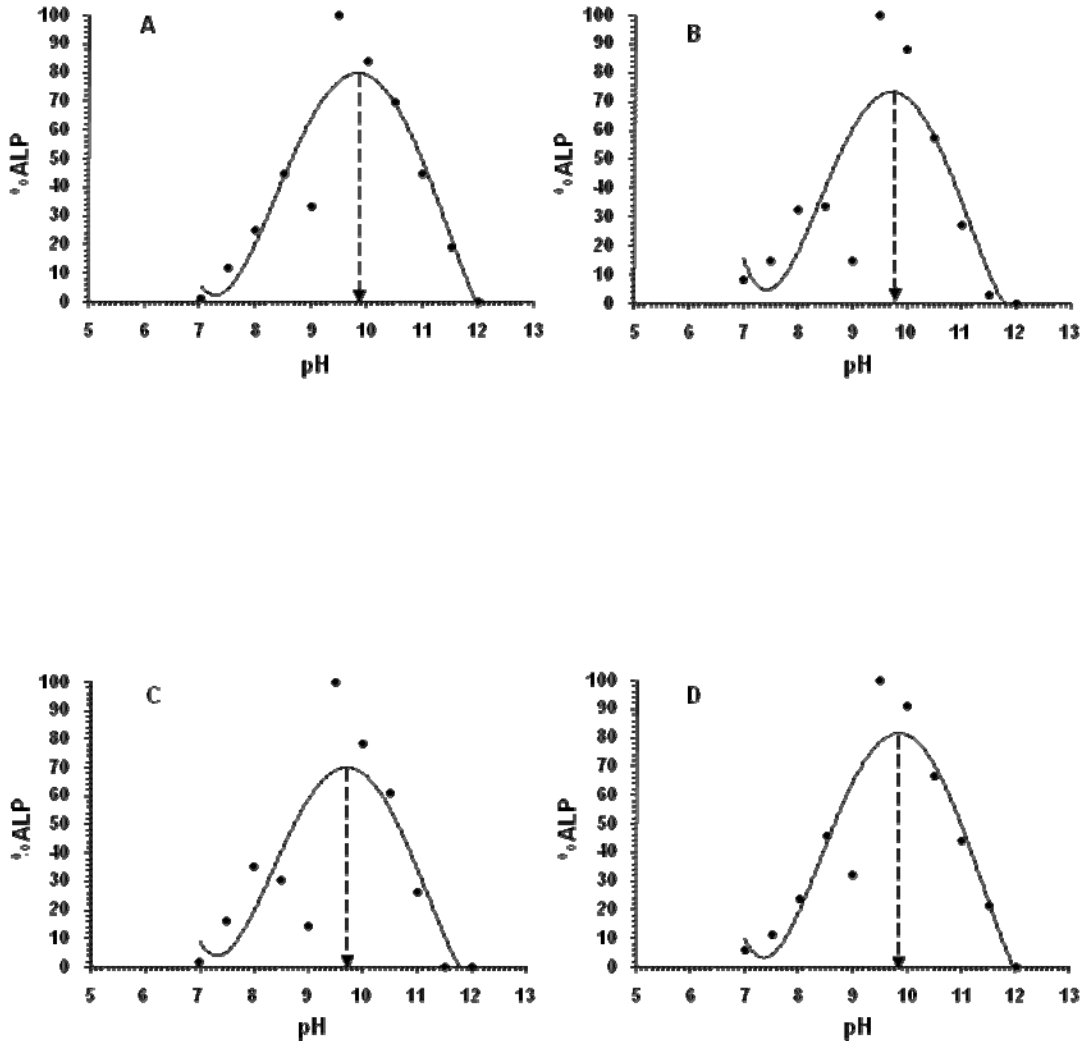


Figure (3.3) Optimum pH on hydatid cyst homogenated ALP (from 4 separate experiments)

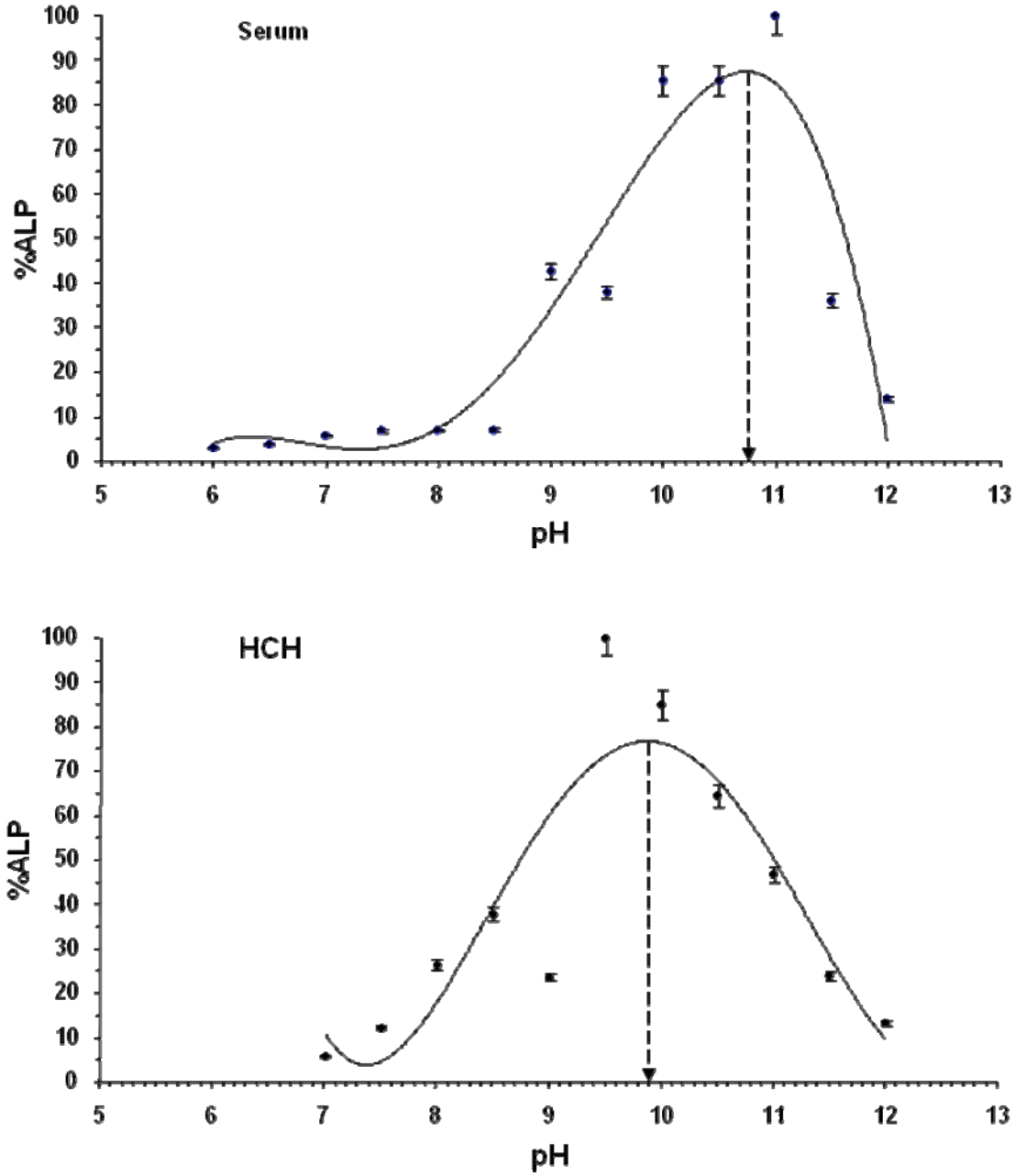


Figure (3.4) pH optimum on serum ALP (results from 6 separate experiments) and HCH alkaline phosphatase (from 4 separate experiments)

3.2.3 Optimum temperature :

As shown in figure 3.5 and figure 3.6, six experiments for serum and 3 for HCH were executed. It is apparent there is two values of optimum temperature, the measured value was shown in figure 3.7, optimum temperature for serum ALP 35.5˚C while the apparent value from the data obtain implies that the temperature from HCH is around 48.7 and as compaired with serum around 40.1, these finding could be of variable biological importance and need to be clearified further. Finally, the optimum temperature for the HCH alkaline phosphatase was higher (figure 3.7) than that estimated for serum ALP (51.8 and 35.3^{o} C for HCH and serum, respectively calculated from the diagram) . To our knowledge, reviewing the literature, no previous study has investigated the optimum temperature of the Echinococcus ALP enzyme.

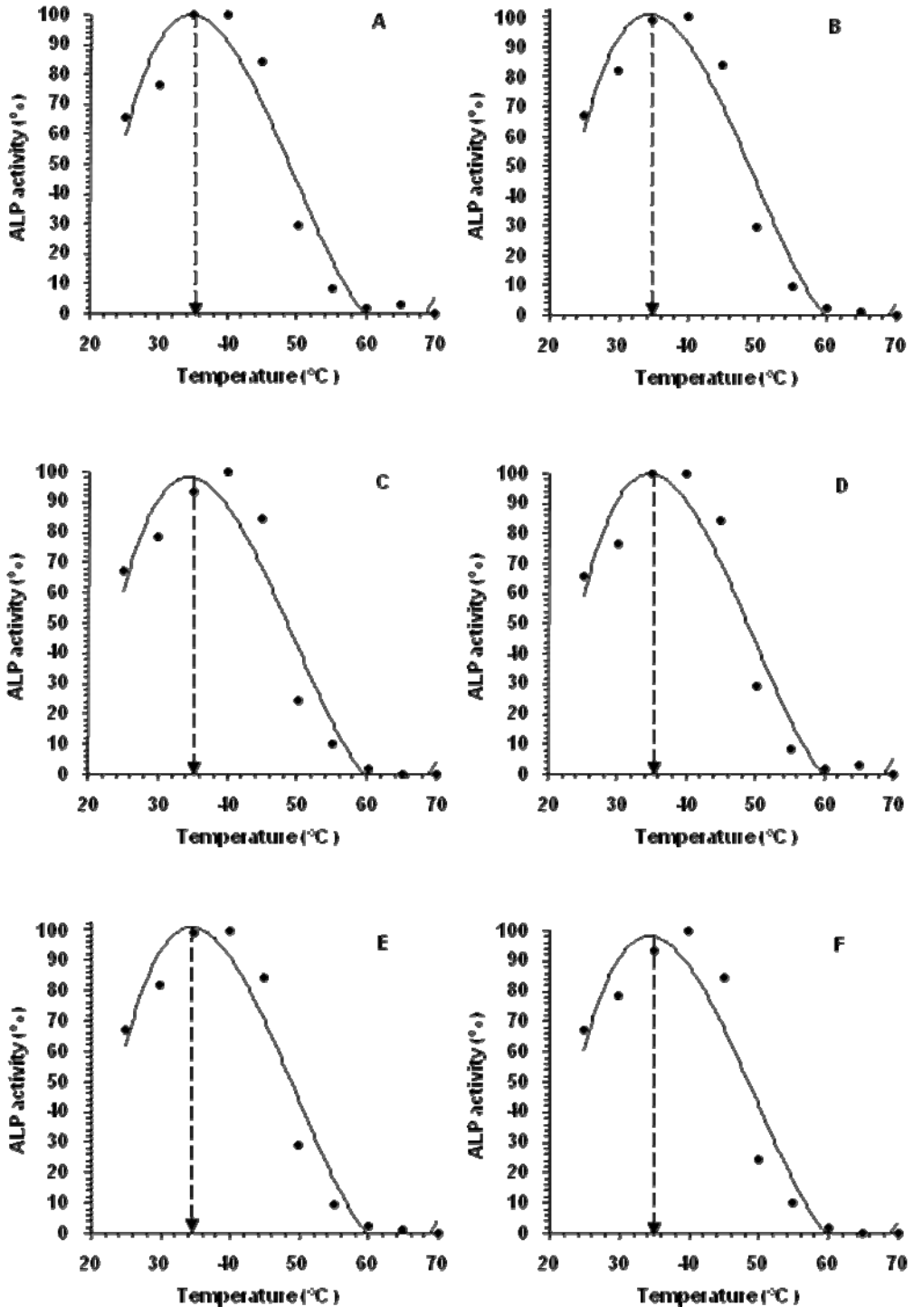


Figure (3.5) Temperature optimum on serum ALP (results from six separate experiments)

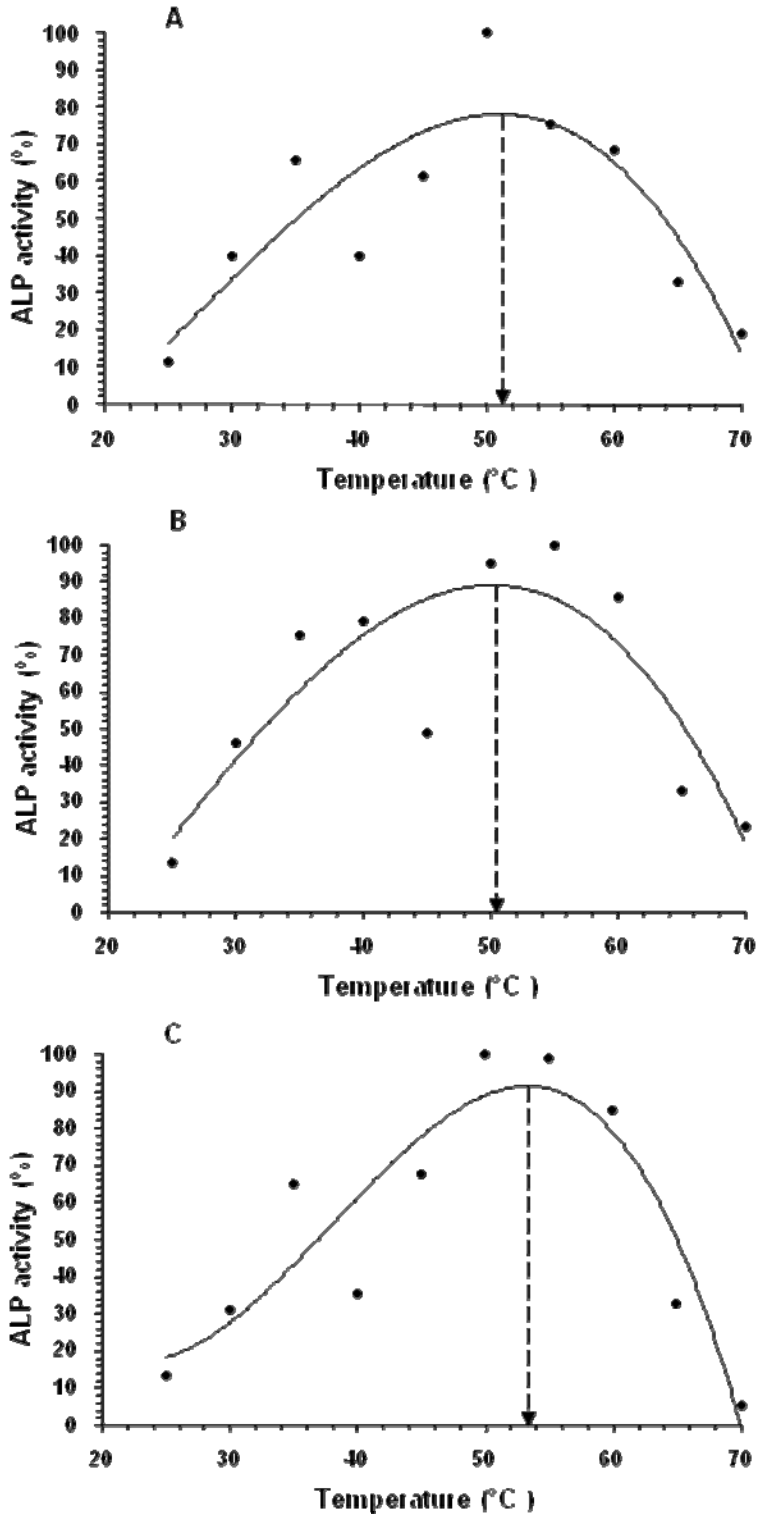


Figure (3.6) Temperature optimum on HCH alkaline phosphatase (results from 3 separate experiments)

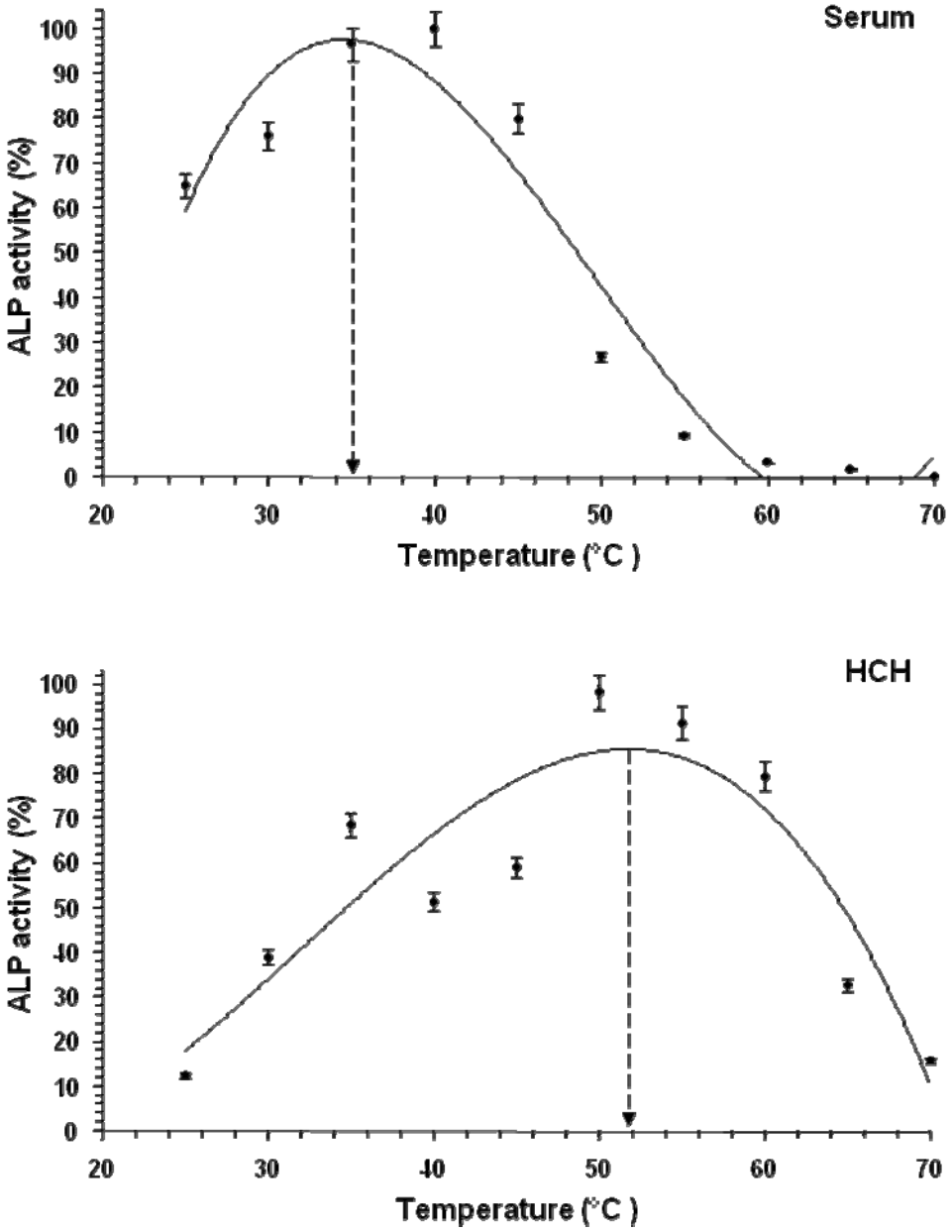


Figure (3.7) Temperature optimum on serum ALP (results from 6 separate experiments) and HCH alkaline phosphatase (from 3 separate experiments)

3.3 Heat inactivation:

Thermostability of serum and parasite ALP enzymes were studied, involving 6 sets for serum (figure 3.8) and another 3 for HCH (figure 3.9). Echinococcus ALP shows a higher resistance to heat inactivation while serum ALP seems to be more heat-labile (figure 3.10), which is more evident at 65^{o}C (ALP remaining activity 1.3% and 44.8% for serum and HCH, respectively).

Vatankhah et al. have stated that the thermal stability of the enzyme from fertile echinococcus HCF is a little more than that of ALP from the sterile HCF and liver enzyme [198]. Moreover, Lawton et al. have proved that the temperature sensitivity of the parasite enzymes, from both E. granulosus and E. multilocularis, displayed a striking resistance to heat denaturation, whereas the liver-type ALP appeared very heat-sensitive, specifically the E. granulosus ALP was more heat resistant than E. multilocularis at 65^{o}C for 30 minutes, while E. multilocularis shows more heat resistance than E. granulosus ALP when the incubation period was extended to 60 minutes [190].

As presented in figure 3.8 and 3.10 the remaining activity of serum ALP at 55^{o}C lies between 61% - 76.6% . This could indicate that the major isoenzyme in our serum sample is the liver ALP isoenzyme[159] , and may add support to our previously mentioned notion, which postulate that serum from patients suffering cholestasis caused by gallstones, associated with high 5'-NT, is representative alternate to human liver-type ALP enzyme .

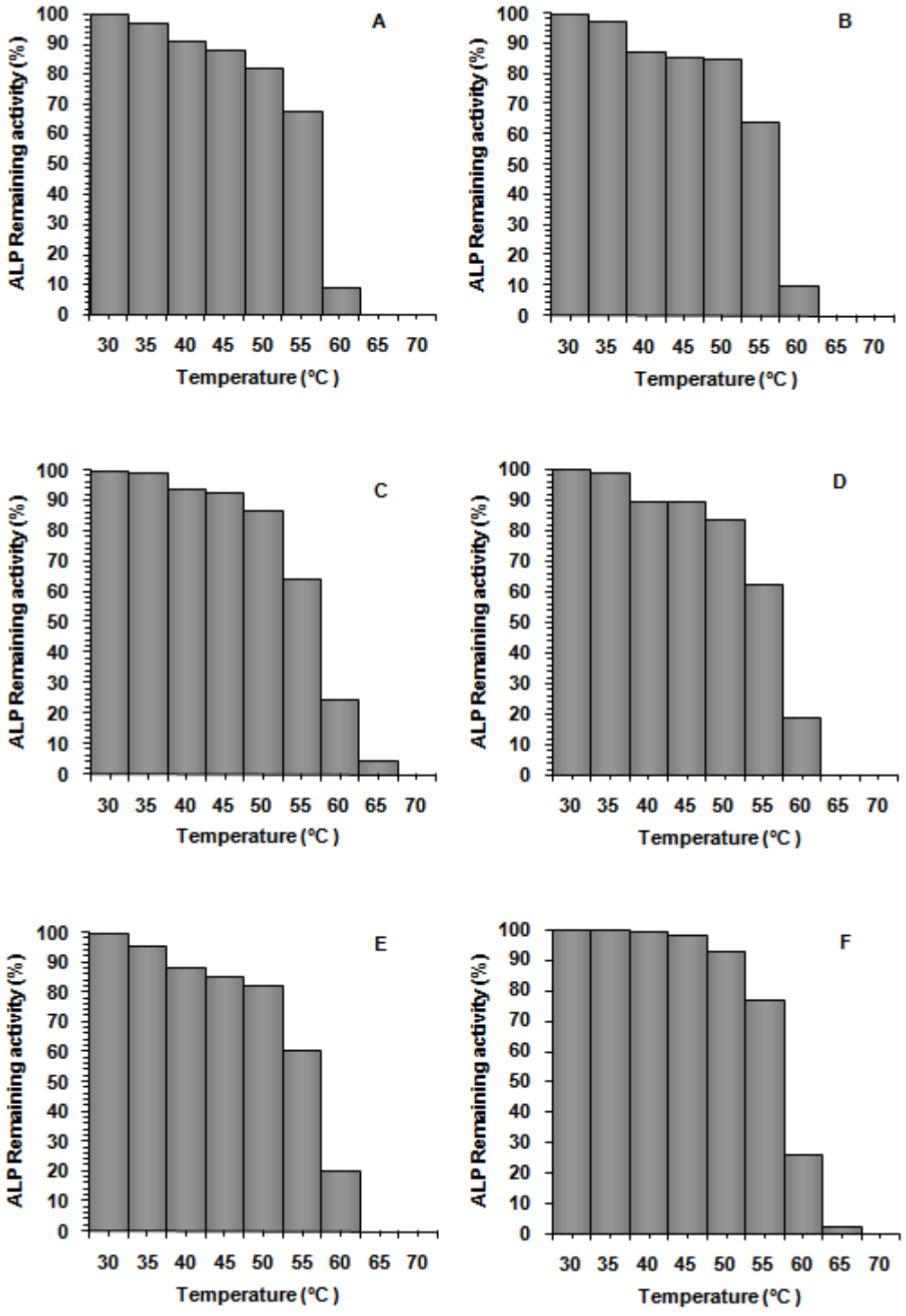


Figure (3.8) Resistance to heat denaturation of serum ALP.

(results from 6 separate experiments)

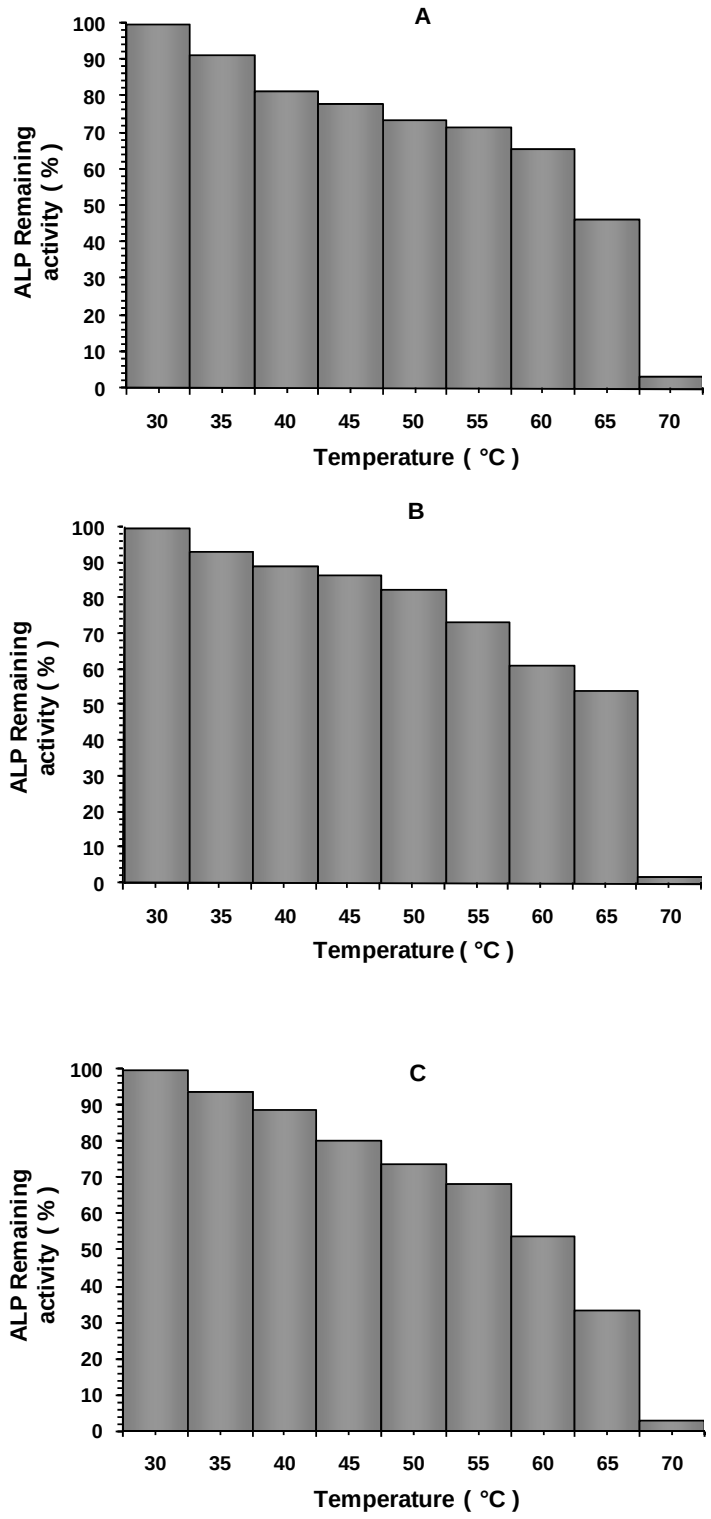


Figure (3.9) Resistance to heat denaturation of HCH alkaline phosphatase (Results from 3 separate experiments)

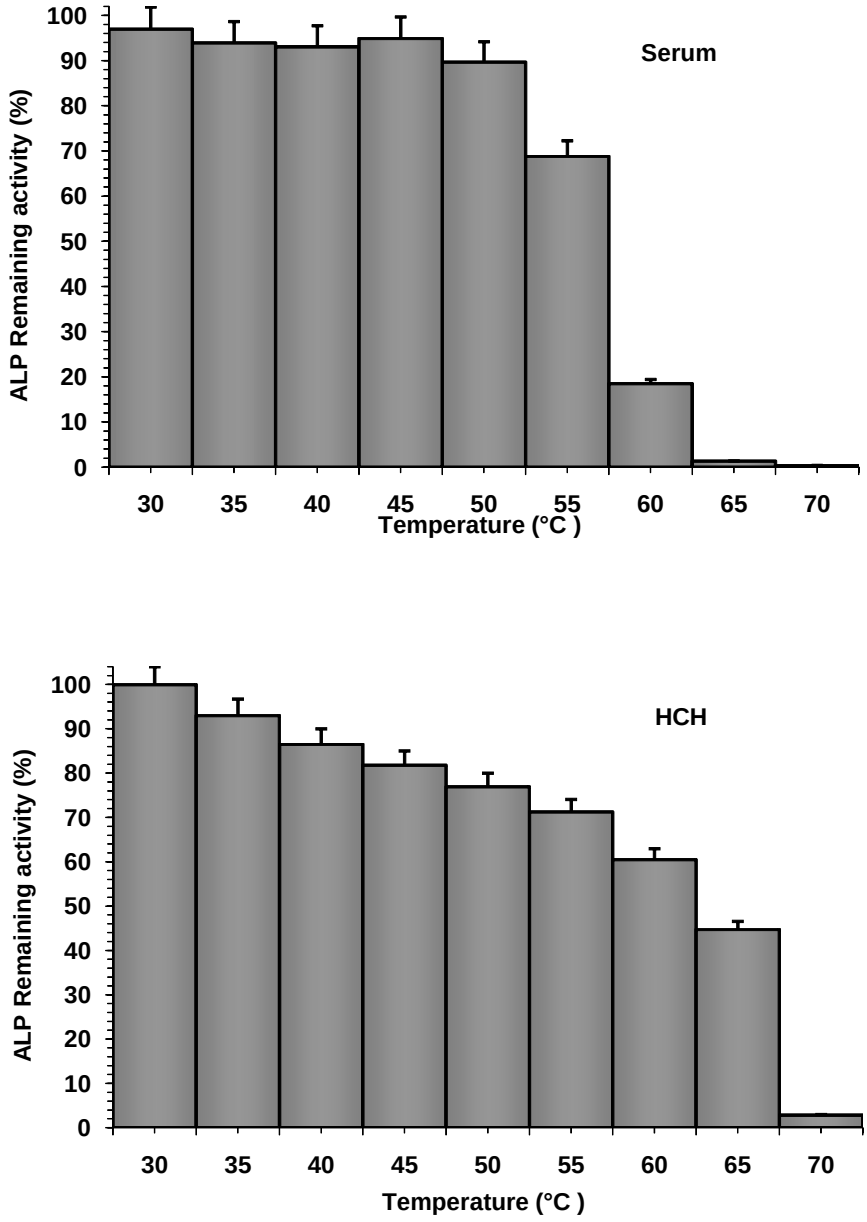


Figure (3.10) Thermostability of serum ALP (Results from 6 separate experiments) and HCH alkaline phosphatase (from 3 separate experiments)

Hence, our results are in consonant with that of Vatankhah and Lowton[198,190]. This Differential thermostability was thought to be due to organ/tissue specific, post-translational modification such as glycosylation[151]. Miura et al.[203] reported that selective removal of the O-linked sugar moieties and sialic acid linkage alters the sensitivity to heat exposure of liver-and bone type ALP.

3.4 L-phenylalanine inhibition:

The inhibitory effect of L-phenylalanine on ALP activity is presented in figure 3.11 and 3.12. Dose-response plot for 6 experiments have shown that serum ALP is inhibited by L-phenylalanine. The calculated IC_{50} from the best fit curve equation, ranges from 49.2-74.1 mM (table 3.3). Data from the 6 experiments were plotted together (figure 3-13) and the mean IC_{50} was calculated as 63.9 mM (table 3.3). No inhibitory effect was observed on ALP from HCH.

The respond of different human ALP isoenzymes to L- phenylalanine have been shown to varied from low sensitive (liver and bone) to highly sensitive (placental and intestinal)[195]. Thus, the wide range of IC_{50} observed herein, can be ascribed to the presence of isoenzymes other than liver type in the serum samples enrolled in this study.

The results of the present study are in agreement with the previous study reporting similar IC_{50} for sterile E. granulosus HCF and liver isoenzyme, with no inhibitory effect on fertile E. granulosus HCF enzyme[198]. Lowton et al.[190], have stated that E. granulosus ALP was

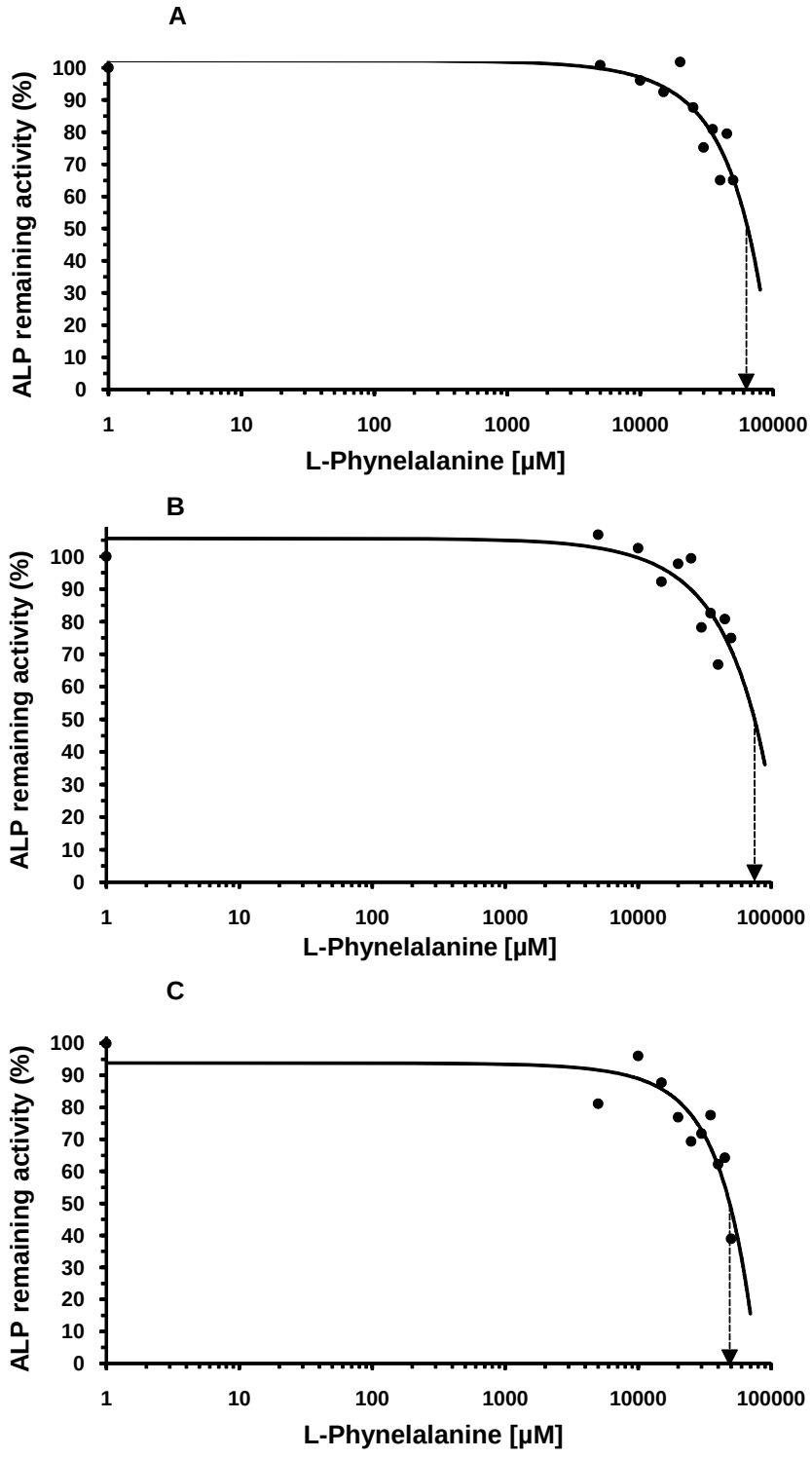


Figure (3.11) Dose respond plot for serum ALP.

The activity was measured at the relevant optimum

pH_{10} (results from 3 separate experiments A,B,C)

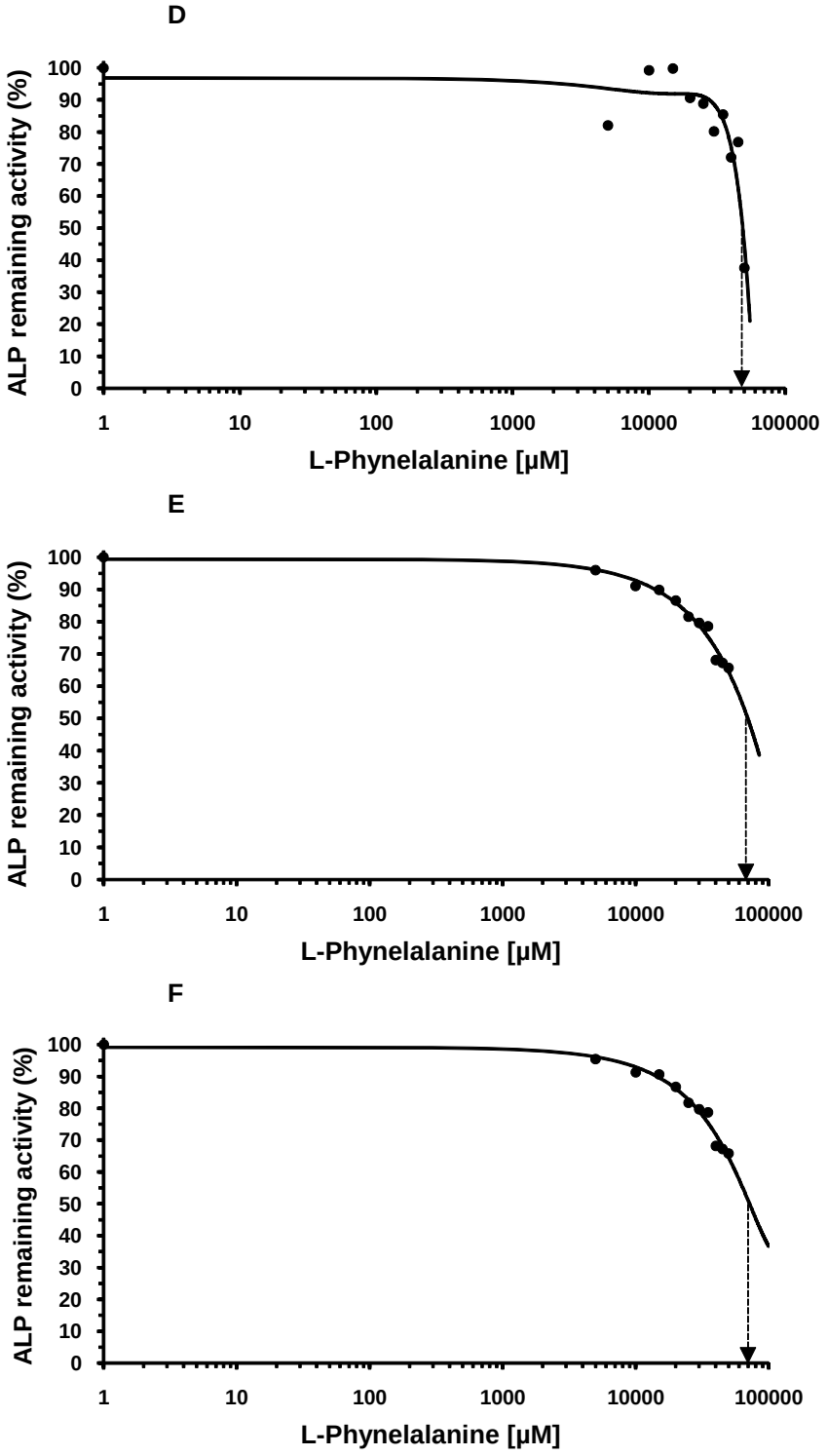


Figure (3.12) Dose respond plot for serum ALP.

The activity was measured at the relevant optimum

pH_{10} (results from 3 separate experiments D,E,F)

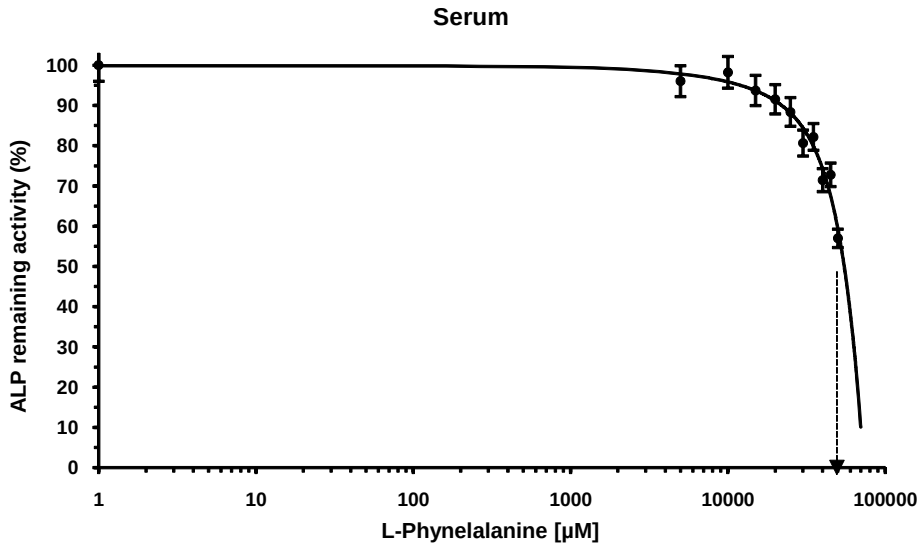


Figure (3.13) Dose respond plot for serum & hydatid HCH alkaline phosphatase.

The activity was measured at the relevant optimum pH_{10}

(A • results from 6 separate experiments A----F)

(B▵ results for HCH)

Table (3.3) Effect of L-phenylalanine on serum ALP activity

Sample	Equation	Km[mM]
A	y = -5E-09x2 - 0.0005x + 102.22	63.8
B	y = -2E-09x2 - 0.0006x + 105.52	74.1
C	y = -1E-08x2 - 0.0004x + 93.87	49.2
D	y = -1E-12x3 + 6E-08x2 - 0.0009x + 96.85	58.3
E	y = 5E-15x3 - 1E-09x2 - 0.0007x + 99.399	66.3
F	y = 4E-14x3 - 4E-09x2 - 0.0006x + 99.115	72.1
Mean	y = -2E-13x3 + 3E-09x2 - 0.0004x + 99.845	63.9

insensitive to L- phenylalanine, E. multilocularis ALP, on the other hand, shows a slight sensitivity to the inhibitor as compared to liver ALP[190].

3.5 Comparative Characteristic of ALP enzymes :

The different characteristic of alkaline phosphatase from hydatid cyst membrane established in our study, involving its affinity to substrate, optimum pH and temperature, as well as its behavior towards heat inactivation and L-phenylalanine inhibition , in comparison with human serum enzyme, revealed that E. granulosus ALP is distinct from human isoenzymes, more specifically from liver ALP isoenzyme.

Moreover, similarity between the characteristic of E. granulosus ALP in our study with that reported by Vatankhah for ALP from E. granulosus hydatid cyst fluid[198]] , as well as those reported by Lawton for E. granulosus, but not for E. multilocularis, may suggest E. granulosus ALP as distinct enzyme from that of E. multilocularis[190].

When Sarciron et al.[93] utilizes pALP of E. multilocularis as the antigen in ELISA test, 100 % of AE patients and 100 % of controls were classified correctly, further more, highly significant differences were obtained between AE and CE patients. The optimal values for discriminating between AE and CE patients results in a sensitivity of 100% and 93% , with a specificity of 82% and 91%, respectively.

Along with the findings of Gottstein who stated that Em2 antigen is not recognized by most of the sera from CE patients [90]. This may indicate

another distinction of E. granulosus from E. multilocularis ALP, namely its immunogenicity . This may support and mandate the need for a specific ELISA test for the specific diagnosis of CE, the major type of echinococcosis in our country, utilizing the E. granulosus pALP. This should be the focus of future study.

2.1 Materials :

2.1.1 Chemicals:

Table (2.1) shows the chemicals used in this work with their suppliers:

Chemicals	*Suppliers*
An hydrous sodium carbonate	BDH, England
sodium bicarbonate	BDH,England
Disodium phenyl phosphate (0.01M)	BDH,England
chloroform	Ferak,Berlin(west)
phenol	BDH,England
Hydrochloric acid (0.1N)	Fluka,Germany
sodium hydroxide 0.5N	Fluka, Germany
potassium ferry cyanide	BDH,England
4- Amino antipyrine	LTD,England
L-phenyl alanine	Fluka,China
Glycine	Fluka,England
sodium chloride	Fluka, Germany
KH_2PO_4	BDH.England
Na_2HPO_4	Fluka, Germany

2.1.2 Instruments:

Table (2.2) shows the instruments and their suppliers;

Instrument	*Suppliers*
1. pH meter	Great lakes (INC)
2. Cool centrifuge	Fanem saopaulo (Brasil)
3. spectrophotometer	Cecil (France)
4. Water bath	Mamert (Germany)
5. Micro pipette	Slamed (Germany)
6. Balance	Saturius lab-Germany
7. MaxiMix	Thermolyne (Iowa)
8. Homogenizer	Potter-S type

2.1.3 Samples:

2.1.3.1 Hydatid cysts:

Hydatid cysts were attained from six hepatic cystic echinococcosis patients attending The Red Crescent Private Hospital, Baghdad, for surgical intervention from January 2007 through August 2008. Based on ultrasonographic examination, only two were identified as having Group 1 CE1 type, three with Group 3 CE4 and one patient with Group3 CE5[94].

Surgical approach in cases of intact liver hydatid cysts include removal of the cysts as whole after injecting 10–20 ml of a scolicidal agent, (10% hypertonic saline), into the intact cyst followed by needle aspiration of the same amount. No formalin was used[57].

The entirely removed cyst membranes were then carefully and thoroughly washed in cold (4°C) saline. The washed membranes were stored in -20°C until further use.

The cyst membranes were cut in small pieces while frozen. One gram of the cyst (wet weight) was suspended in 3ml of 100mM Tris-HCl pH 7.6, 100mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 0.02mM $ZnCl_2$, and homogenized using a potter-S type homogenizer, equipped with an ice bath, as a coolant to a void the rise in the system temperature, for 30 minutes to ensure complete homogenization. The homogenate was centrifuged at 5000 r.p.m for 15 min using Fanemsaopaulo, Brasil cool centrifuge[93]. Supernatant pooled together and divided into into aliquots and stored at frozen(-20°C) until further analysis.

2.1.3.2 Serum:

Fasting venous blood samples were acquired from patients with professional diagnosis of gallstones, attending the above hospital for surgical treatment. Serum was separated, 5'-nucleotidase activity was measured to confirm that the raised ALP activity is mainly due to liver isoenzyme[159].

Collected serum samples were pooled together until further analysis.

2.2 Methods:

2.2.1 Measurement of alkaline phosphatase:

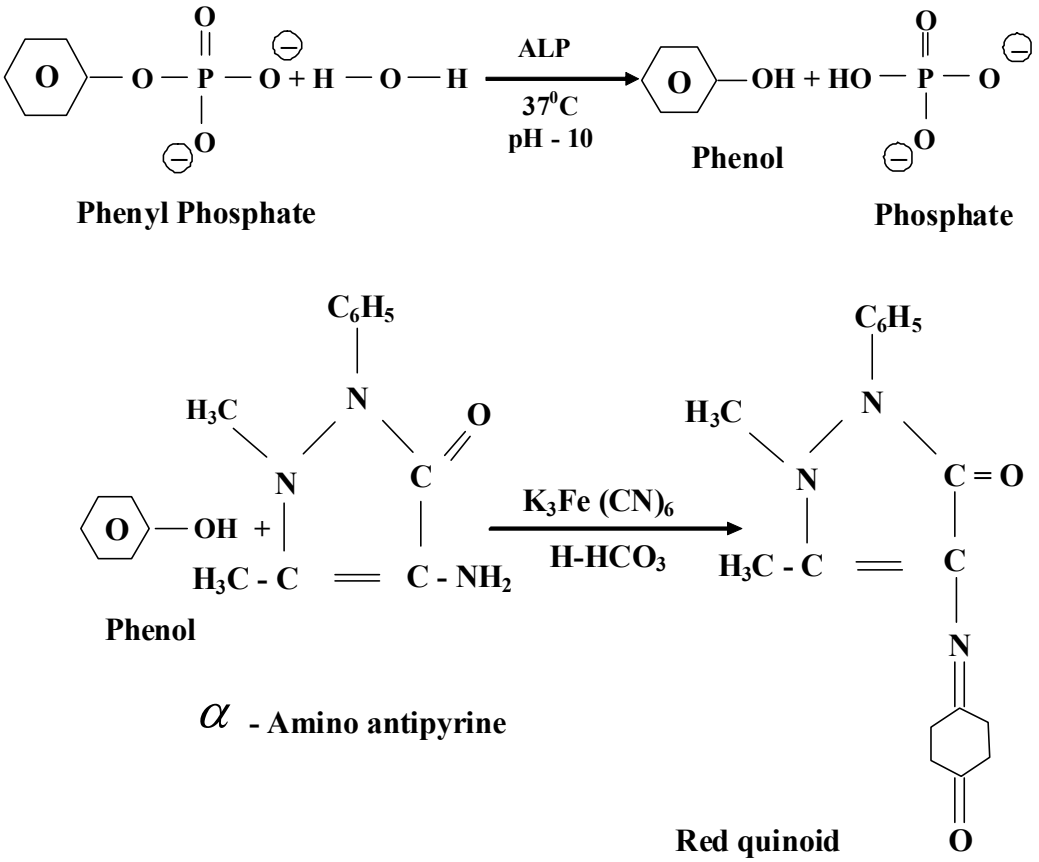
Alkaline phosphatase activity in serum and cyst homogenate were assayed according to the method of kind and king[200].

When assay samples were kept at room temperature, ALP activity usually shows a slight but real increase in activity within four hours. In frozen samples, there is a decrease in activity, which is slowly recovered after thawing . The cause of this phenomenon is not known, but may be due to renaturation of partially denatured enzyme or to dissociation, upon warming, of a multimer of the enzyme that was formed in the freeze process[136]. Consequently, frozen samples, serum and cyst homogenate, were thawed and left at room temperature for 6-8 hours to achieve full ALP activation.

2.2.1.1 Principle:

α-amino antipyrine reacts with phenolic substance, which is liberated from disodium phenyl phosphate by the action of the enzyme ALP , in the presence of alkaline oxidizing agent to produce quinonoid substitution products . This give a red color proportional to the amount of the liberated phenol , which can be determined colorimetrically.

$$\text{Phenyl phosphate} \xrightarrow[\text{pH 10}]{\text{Alkaline phosphatase}} \text{Phenol} + \text{phosphate}$$



2.2.1.2 Reagents:

1- Bicarbonate buffer (0.1M, pH 10): 6.36 g of anhydrous sodium carbonate and 3.3 g of sodium bicarbonate were dissolved in 500 ml of DW, adjusted to pH 10 using pH meter. The volume was made up to 1L with DW. the buffer was kept at 4˚C until time of use.

2- Substrate (0.01M disodium phenyl phosphate) : 2.18 g of disodium phenyl phosphatewas dissolved in one liter of distilled water. The

solution was brought quickly to boiling to kill any organism, and then was cooled immediately and preserved by adding 4 ml chloroform, and was kept in (4.8˚C).

3-Hydrochloric acid (0.1 N) : 8.35 ml of concentrated HCl (37% , Sp.gr 1.18) was diluted to 1L with DW.

4-Stock phenol standard (1 mg/ml): 1.0 g pure crystalline phenol was dissolved in 1 liter of 0.1N HCl and the solution was kept at 4˚C in a brown bottle.

5- Working phenol standard (1 mg/100 ml): 1 ml of stock phenol standard was diluted to 100 ml with DW. Then preserved with a few drops of chloroform, and kept at 4^{o}C in a brown bottle.

6-Sodium hydroxide(0.5 N): 20 g of NaOH was dissolved in DW and diluted to 1L.

7-Sodium bicarbonate (0.5 N): 42 g of $NaHCO_3$ was dissolved in DW and diluted to 1 L.

8- α-amino antipyrine(0.6%): 6 g of α-amino antipyrine was dissolved in DW and diluted to 1 L and then stored in a brown bottle.

9- Potassium ferricyanide (2.4%) : 24 g of $K_3[Fe(CN)_6]$ was dissolved in DW and diluted to 1L then the solution was stored in a brown bottle.

2.2.1.3 <u>Procedure:</u>

The following addition were made into the respective labeled tubes:

Test (T) : in a test tube , 1 ml of 0.1M sodium bicarbonate buffer pH 10 was mixed with 1ml of 0.01M disodium phenyl phosphate and placed in a water bath at 37°C for three minutes, 0.1ml of sample (serum or cyst homogenate) was added, mixed and incubated for exactly 15 minutes. The tube was removed from the water bath and 0.8ml of 0.5N sodium hydroxide was immediately added and mixed well.

Control(C) : in a test tube, 1ml of buffer , 1ml of substrate and 0.8ml of 0.5N sodium hydroxide was mixed together and then 0.1ml of sample (serum or cyst homogenate) was added and mixed .

Standard (S) : in a test tube, 1ml of phenol working standard (1mg/100ml) was mixed with 1.1ml of buffer and 0.8ml of 0.5N sodium hydroxide.

Blank (B) : in a test tube, 1.1ml of buffer , 1ml of distilled water and 0.8ml of 0.5N sodium hydroxide were mixed .

To all tubes, 1.2ml of 0.5N sodium bicarbonate was added, followed by addition of 1ml of 0.6% α-amino antipyrine and 1ml of 2.4% potassium ferricyanide solution. Each tube was mixed well after each addition . The successive additions adjust the pH and develop the reddish-brown color which is then measured at 510nm using spectrophotometer.

Note: All analysis were carried out in multiples. The mean was calculated and use in calculation.

2.2.1.4 Calculation:

The amount of phenol present in the standard tube is 10 µg . Thus the phenol produced in 15 minutes in the test tube is:

(T-C) / (S-B) X 10 µg

Hence, the mg of phenol liberated by 100ml of serum would be:

(T-C) / (S-B) X 10 mg

Since one king-Armstrong unit(K.A.U) is the 1mg of phenol liberated by 100ml of serum in 15 minutes under the conditions of the test.

The activity of ALP in assayed samples then:

ALP(K.A.U) = (T-C) / (S-B) X 10

2.2.2 Factors affecting ALP activity:

2.2.2.1 Substrate Concentration:

For a typical enzyme, as substrate concentration is increased, the initial velocity (v_i) of the reaction increases until it reaches a maximum value V_{max} (Figure 2.1). When further increases in substrate concentration do not further increase v_i, the enzyme is said to be "saturated" with substrate. The shape of the curve that relates activity to substrate concentration (Figure 2.1) is hyperbolic[201].

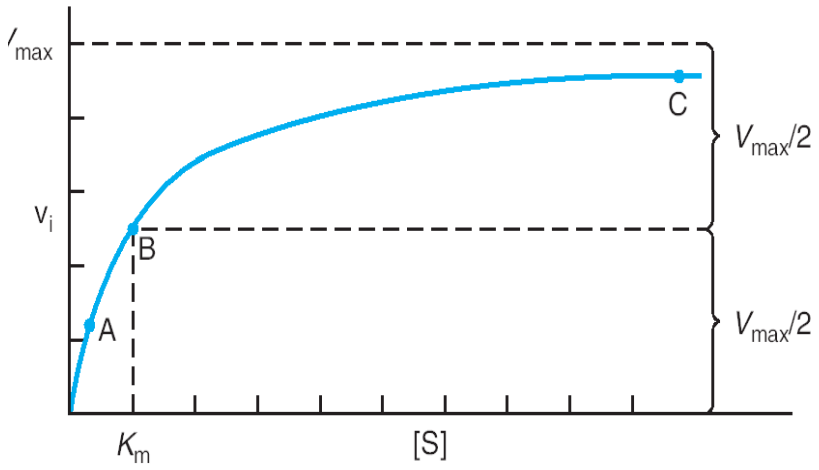


Figure (2.1) Effect of substrate concentration on the initial velocity of an enzyme –catalyzed reaction [201]

The Michaelis constant (K_m) is the substrate concentration at which v_i is half the maximal velocity ($V_{max}/2$) attainable at a particular concentration of enzyme. K_m thus has the dimensions of substrate concentration.

The direct measurement of the numeric value of V_{max} and therefore the calculation of K_m often requires impractically high concentrations of substrate to achieve saturating conditions. A linear form of the Michaelis-Menten equation circumvents this difficulty and permits V_{max} and K_m to be extrapolated from initial velocity data obtained at less than saturating concentrations of substrate. Starting with the equation

$$v_i = \frac{V_{max}[S]}{K_m + [S]}$$

By inversion;

$$\frac{1}{v_1} = \frac{K_m + [S]}{V_{max}[S]}$$

or

$$\frac{1}{v_i} = \frac{K_m}{V_{max}[S]} + \frac{[S]}{V_{max}[S]}$$

Which can be expressed simply as ;

$$\frac{1}{v_i} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

The latter is the equation for a straight line, y = ax+ b, where y = 1/ v_i and x = 1/[S]. A plot of 1/ v_i as y as a function of 1/[S] as x therefore gives a straight line whose y intercept is 1/ V_{max} and whose slope is K_m / V_{max}. Such a plot is called a double reciprocal or Lineweaver-Burk plot[201] (Figure 2.2). Setting the y term of the above equation equal to zero and solving for x reveals that the x intercept is −1/ K_m.

$$0 = ax + b; \text{ therefore, } x = \frac{-b}{a} = \frac{-1}{K_m}$$

K_m is thus most easily calculated from the x intercept.

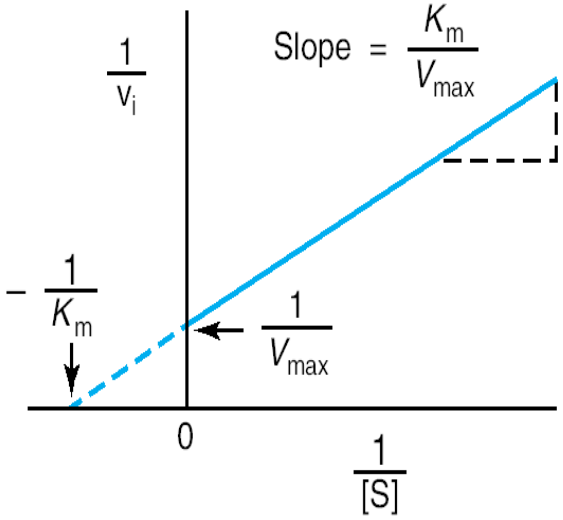


Figure (2.2) Double reciprocal or Lineweaver-Burk plot of $1/V_i$ versus $1/[S]$ used to evaluate K_m and V_{max} [201].

2.2.2.1.1 <u>Reagent:</u>

1. Substrate stock solution (0.04M disodium phenyl phosphate): 8.72g of disodium phenyl phosphate was dissolved in 500 ml DW. and diluted to 1L , boiled for 1min to kill any organism .Cooled and preserved with 4ml chloroform , stored at 4˚C.

2. Working substrate solutions: different concentrations of working substrate solutions ***(0.001, 0.002, 0.003, 0.004, 0.005, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035, 0.04M)*** were prepared by diluted the following volume in 25 ml DW ***(0.5, 1, 1.5, 2, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20ml).***

2.2.2.1.2 <u>Procedure:</u>

ALP activity was measured in serum and cyst homogenate as previously described using the above substrate working solutions.

2.2.2.2 <u>Temperature :</u>

Raising the temperature increases the rate of both uncatalyzed and enzyme-catalyzed reactions by increasing the kinetic energy and the collision frequency of the reacting molecules. However, heat energy can also increase the kinetic energy of the enzyme to a point that exceeds the energy barrier for disrupting the noncovalent interactions that maintain the enzyme's three-dimensional structure[201].

The polypeptide chain then begins to unfold, or denature, with an accompanying rapid loss of catalytic activity. The temperature range over which an enzyme maintains a stable, catalytically competent conformation depends upon—and typically moderately exceeds—the normal temperature of the cells in which it resides. Enzymes from humans generally exhibit stability at temperatures up to 45–55 °C[202].

2.2.2.2.1 Procedure:

ALP activity was measured in serum and cyst homogenate as previously described with different incubation temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75˚C).

2.2.2.2.2 Calculation:

ALP activity was calculated as previously described , and results were expressed as a percentage of the highest activity.

2.2.2.3 Hydrogen ion Concentration (pH):

The rate of almost all enzyme-catalyzed reactions exhibits a significant dependence on hydrogen ion concentration Most intracellular enzymes exhibit optimal activity at pH values between 5 and 9. The relationship of activity to hydrogen ion concentration (Figure 2.3) reflects the balance between enzyme denaturation at high or low pH and effects on the charged state of the enzyme, the substrates, or both[202].

2.2.2.3.1 <u>Reagents:</u>

1. Mono potassium phosphate KH_2PO_4(0.067M): 4.559 g of KH_2PO_4 was dissolved in 200ml DW and completed to a final volume 500 ml.

2. Disodium phosphate Na_2HPO_4(0.067M): 4.753 g of $Na_2HPO_4$was dissolved in 200ml DW and completed to a final volume 500 ml.

3. Working phosphate buffers(0.067M): Phosphate buffers with the following pH values were prepared by taking the volume of (0.067M) KH_2PO_4 solution specified in the following table and completed with (0.067M) Na_2HPO_4 solution to 1 liter. The prepared buffers were adjusted using pH meter.

pH	ml. of KH_2PO_4
6	878
6.5	682
7	389
7.5	159
8	68

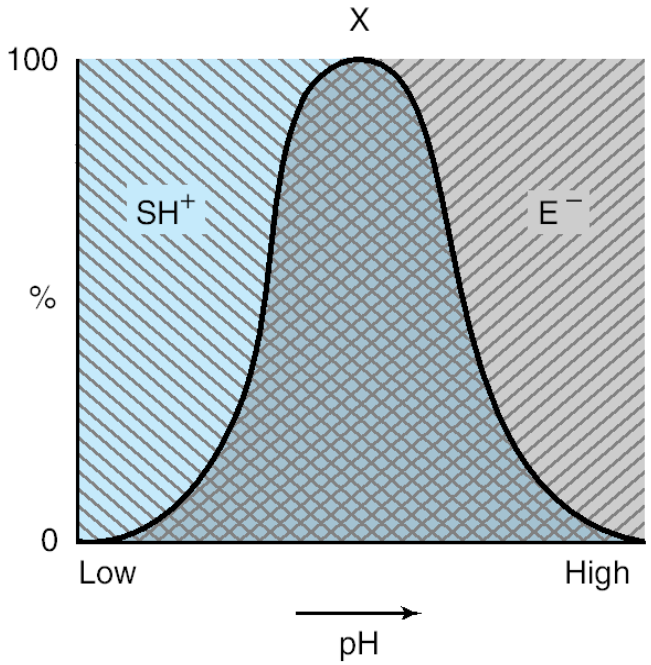


Figure (2.3) Effect of pH on enzyme activity. Consider, for example, a negatively charged enzyme (EH^-) that binds a positively charged substrate (SH^+). Shown is the proportion (%) of SH^+ and of EH^- as a function of pH. Only in the cross-hatched area do both the enzyme and the substrate bear an appropriate charge[202].

4. Glycine-Sodium Chloride mixture, (0.1M) of each: 8.759 g Glycine and 2.922 g NaCl were dissolved in 200 ml DW and completed to 500 ml.

5. Sodium Hydroxide NaOH (0.1M) : 2 g of NaOH was dissolved in 200 ml DW and completed to 500 ml .

6. Working Glycine-NaOH buffers(0.1M): buffers with the following pH values were prepared by taking the volume of Glycine-Sodium Chloride mixture specified in the following table and completed with NaOH solution(0.1M) to 1 L . The prepared buffers were adjusted using pH meter.

pH	ml. of Glycine NaCl mixture
8.5	965
9	885
9.5	790
10	630
10.5	561
11	510
11.5	497
12	462

2.2.2.3.2 <u>Procedure:</u>

ALP activity was measured in serum and cyst homogenate as previously described with the above working buffer solutions.

2.2.2.3.3 Calculation*:*

ALP activity was calculated as previously described, and results were expressed as a percentage of the highest activity.

2.2.3 Heat Stability:

The differential thermo stability of enzymes is thought to be due to organ/tissue specific, post-translational modifications such as glycosylation [193]. This characteristic can be examined by exposing the enzyme, contained in assay samples, to different temperature after which, the residual activity of the enzyme will be measured.

2.2.3.1 Procedure:

1- Samples of serum or cyst homogenate were incubated at different temperature (25,30,35,40,45,50,55,60,65,70) for 30 min in a water bath .
2- Samples were immediately cooled to room temperature and ALP activity was measured as described above.

2.2.3.2 Calculation:

The residual activity measured was expressed as a percentage of the original activity of the sample.

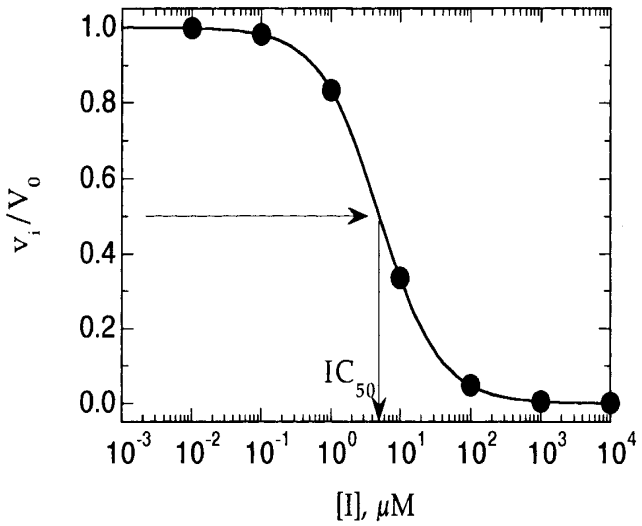
2.2.4 Inhibition by L-phenylalanine:

Dose—response plots (figure2.4) can be used to follow the effects of an inhibitor on the initial velocity of an enzymatic reaction at a fixed concentration of substrate. The concentration of inhibitor required to achieve a half-maximal degree of inhibition is referred to as the IC_{50} value (for inhibitor concentration giving 50% inhibition), and the equation describing the effect of inhibitor concentration on reaction velocity is related to the Langmuir isotherm equation as follows:

$$\frac{v_i}{v_0} = \frac{1}{1 + \frac{[I]}{IC_{50}}}$$

Where v_i is the initial velocity in the presence of inhibitor at concentration [I] and v_0 is the initial velocity in the absence of inhibitor The term v_i/v_0 in the equation above is referred to the fractional activity remaining at a given inhibitor concentration

Dose—response plots are widely used for comparing the relative inhibitor potencies of multiple compounds for the same enzyme, under well-controlled conditions. The method is popular because it permits analysts to determine the IC_{50} by making measurements over a broad range of inhibitor concentrations at a single, fixed substrate concentration. The IC_{50} value of a particular inhibitor can change with changing solution conditions, so it is very important to report the details of the assay conditions along with the IC_{50} value. For example, in the case of competitive inhibition, the IC_{50} value observed for an inhibitor will depend on the concentration of substrate present in the assay, relative to the K_m of that substrate.



Figure(2.4)Dose-response plot of enzyme fractional activity as a function of inhibitor concentration . Note that the inhibitor concentration is plotted on a log scale. The value of the IC_{50} for the inhibitor can be determined graphically as illustrated[202].

2.2.4.1 Reagents:

Stock bicarbonate buffer (0.01M, pH10)-L-phenylalanine (50 mM) mixture: 0.663 g of L-phenylalanine was dissolved in 30 ml of bicarbonate buffer (0.01M).The pH was adjusted to 10 using pH meter. The volume was made up to 50 ml with bicarbonate buffer. The mixture was kept at 4˚C until time of use.

2.2.4.2 Procedure:

1. Working buffers containing different concentrations of L-phenylalanine were prepared as indicated in the following table

[phenylalanine]	Buffer+phenylalanine	Buffer (only)
0 mM	0 ml	1 ml
5 mM	0.1 ml	0.9 ml
10 mM	0.2 ml	0.8 ml
15 mM	0.3 ml	0.7 ml
20 mM	0.4 ml	0.6 ml
25 mM	0.5 ml	0.5 ml
30 mM	0.6 ml	0.4 ml
35 mM	0.7 ml	0.3 ml
40 mM	0.8 ml	0.2 ml
45 mM	0.9 ml	0.1 ml
50 mM	1 ml	0 ml

2. Duplicate samples of serum and cyst homogenate were preincubated for 10min with the above working buffers in a water bath at 37˚C.

3. The following steps were done as previously described for the measurement of ALP activity.

2.2.4.3 Calculation:

The IC_{50} were graphically determined from the relevant curves with different inhibitor concentrations.

Curriculum Vitae

Name: Mariam Fadhil Nassir
Date of birth: 18/8/1980
Nationality: Iraqi
Nationality ID No: 320795
Social States: Married
Sex: Female
Religion: Christian
Tel: +964 7901195568
Adress: Iraq/Baghdad, Alhabibia, Q.9 April, N. 734 / S. 15 /B. 36
e-mail: Mary_fns@yahoo.com
Certificates: B.SC.in chemical scientific granuated in 2002 from Al-mostansuria University
Occup: Repeater
Office: Kindy College of Medicine
Date of Disscusion: 28/5/2009
Supervisor: Assist.Prof. Dr. Salman A. Ahmed
Name of Project: Comparative characterization and biochemical analysis of alkaline phosphatase from Echinococcus granulosus cysts and Human serum

"Dedication"

To

My loved husband "Jan" & sweetie daughter "lourd" who gave me the light to my life.

My parents & my husband's parents whom were generous in their lover & help.

My brother's family who are faraway from me but housing in my heart.

Nour & Rafi who giving me the support and encouragement.

Mariam

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List of abbreviations

No.	*Abbreviation*	*Meaning*
1	ALP	Alkaline phosphatase
2	α	alpha
3	AE	Alveolar echinococcosis
4	B	Blank
5	CBN	Commission on biochemical nomenclature
6	C	Control
7	CE	Cystic echinococcosis
8	CL	Cystic lesion
9	CNS	Central Nervous System
10	CT Scan	Computerized Tomography
11	C^o	Degree centigree
12	DNA	Deoxyribonucleic acid
13	DW	Distilled water
14	E	Echinococcus
15	EDTA	Ethylene diamine tri tetra acetic acid
16	ELISA	Enzyme-linked immunosorbent assay
17	EmALP	Echinococcus multilocularis alkaline phosphatase
18	EmC-Ag	Echinococcus multilocularis crudeantigen
19	Em2	Echinococcus multilocularis antigen 2
20	g	gram
21	HCF	Hydatid cyst fluid
22	HCH	Hydatid cyst homogenated
23	HD	Hydatid disease
24	IALP	Intestinal alkaline phosphatase
25	IUPAC-IUB	International union of pure and applied chemistry – International union of Biochemistry
26	K.A.U	King Armstrong Unit
27	Km	Michaelis constant
28	L	Liter
29	M	Molarity
30	µg	Microgram
31	mg	milligram
32	min	minute

33	ml	Milliliter
34	mM	millimole
35	MABG11	Murine monoclonal antibody
36	MRI	Magnatic Resonance imaging
37	N	Normality
38	ND	Not detected
39	Nm	nanometer
40	5'-NT	5'-nucleotidase
41	pALP	Purified alkaline phosphatase
42	pALP-Ag	Purified alkaline phosphatase antigen
43	pH	Hydrogen ion concentration
44	RBC	Red blood cells
45	r.p.m	(revolution) round per minute
46	S	Standard
47	[S]	Substrate concentration
48	T	Test
49	Tris	Tri (Hydroxy methyl) methylamine
50	V_i	Initial velocity
51	V_{max}	Maximal velocity
52	WHO	World health organization

Republic Of Iraq
The Ministry Of higher Education
And Scientific Research
Al-Nahrain University
College Of Science
Department Of Chemistry

Comparative Characterization And Biochemical Analysis Of Alkaline Phosphatase From Echinococcus Granulosus Cysts And Human Serum

A Thesis
Submitted to the College of science
Of Al- Nahrain University
In Partial Fulfillment of the Requirements
For the degree of Master of Science
In
Clinical Biochemistry

By
Mariam Fadhil Nassir
B.Sc. in chemistry (Al-mostansuria University 2002)

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SUMMARY

Background:

Hydatidosis, a zoonitic parasitic disease, is one of the most important economic and health problem in Iraq and many other countries in the Middle East. It is caused by Echinococcus Multilocularis and Echinococcus granulosus, the larval stage of which is localized in the liver, lungs and other visceral organs of intermediate hosts such as sheep, goats and cattle.

Human are considered to be an accidental intermediate host, either by eating improperly disinfected vegetables or by close contact with dogs. The mature form lives in the ileum of candies as its definitive hosts, where it leads to an asymptomatic infection .

Serology or immunodiagnostic tests are the most useful techniques for the detection of hydatid cysts in human patients.

Objectives:

One of the reasons why Echinococcus granulosus cysts are described as a public health problem is the lack of effective chemotherapy.

Reviewing the literature bears in mind the possibility of an effectual and successful chemotherapeutic treatment in the early stages of cyst development, as indicated by many reported studies. Thus, the major problem in the management of Hydatid disease seems to be related to the lack of an efficient routine diagnostic test capable of identifying asymptomatic patients at early stages of the disease. Consequently, the need

for a consistent diagnostic test, applicable for mass screening in hyperendemic regions, has become mandatory.

Much attention has been focused on the multilocularis species which represent the major type affecting western countries. These cumulative efforts have recently evolved a commercially available and reliable immunodiagnostic test for the specific diagnosis of Alveolar ehinococcus. Unfortunately, the test fails to identify most of the patients suffering from cystic echinococcosis, caused by the closely related echinococcus granulosus.

Hence, this will detract from the valuablity of the test in districts, where echinococcus granulosus is well known as the main endemic type, including Iraq, and necessitating the requirement for further studies with regard to echinococcus granulosus.

The present study was conducted to estimate the kinetic parameters and biochemical characteristics of alkaline phosphatase in fertile and sterile cyst membranes from patients with hepatic cystic echinococcosis. The raised alkaline phosphatase serums of patients suffering cholestasis caused by gallstones were used as a representative alternate to Human liver- type alkaline phosphatase. Comparisons were aimed to reveal whether these two cyst membranes enzymes are different from each other and from the Human liver- type alkaline phosphatase.

Subject and methods:

In this study two samples were used, the cyst and human serum.

Hydatid cysts were obtained from six hepatic cystic echinococcosis

patients attending The Red Crescent Private Hospital, Baghdad, for surgical intervention from January 2007 through August 2008. Based on ultrasonographic examination, only two were identified as fertile, three were sterile and one patient with calcified cyst.

Fasting venous blood samples were acquired from patients with professional diagnosis of gallstones, attending the above hospital for surgical treatment. Serum was separated, 5'-nucleotidase activity was measured to confirm that the raised alkaline phosphatase activity is mainly due to liver isoenzyme

Collected serum samples were pooled and divided into aliquots, stored at -2 C until further analysis.

The present study was measurement of alkaline phosphatase activity in serum and cyst homogenate according to the method of kind and king.

Kinetic studies will involve the affinity of these enzymes to their substrate, as well as the effect of other factors including hydrogen ion concentration (pH) and temperature. Heat inactivation and the effect of specific inhibitor, namely L-phenylalanine, are also to be examined on the activity of the three alkaline phosphatase enzymes.

Results:

1. Different alkaline phosphatase activity were observed in fertile cysts while there was no alkaline phosphatase activity in hydatid cyst homogenated from sterile cysts and still not able to be detected even when larger volume of the hydatid cyst homogenated sample were used for the estimation and can be explained that by the presence or

the absence of fresh protoscoleces in fertile or sterile hydatid cyst, respectively. Because of this statement it can be differentiate between the sterile & fertile cyst.

2. By the results of the biochemical characterization of alkaline phosphatase, there were different Km values, optimum pH and optimum temperature for each hydatid cyst homogenated & human-liver type.
3. The heat-sensitive specifically showed that the echinococcus granulosus alkaline phosphatase was more heat resistance than echinococcus multilocularis & liver-type enzyme.
4. The respond of different human alkaline phosphatse isoenzymes to L-phenylalanine have been shown to varied from low sensitive (liver and bone) to highly sensitive (placental and intestinal). Thus, the wide range of IC_{50} observed herein, can be ascribed to the presence of isoenzymes other than liver type in the serum samples enrolled in this study. No inhibitory effect was observed on alkaline phosphatase from hydatid cyst homogenate.

Conclusion:

Considering present findings along with the following discussion, the following conclusions might be derived:

1. Echinococcus granulosus Alkaline Phosphatase serve as a marker of cyst viability.
2. The different characteristic of alkaline phosphatase from hydatid cyst membrane revealed that echinococcus granulosus Alkaline phosphatase is distinct from human isoenzymes more specifically from liver alkaline phosphatase isoenzyme.

Supervision Certificate

I certify that this thesis was prepared under my supervision at the department of chemistry, college of science, Al-Nahrine University as a partial fulfillment of the requirements for the degree of Master of Science in Clinical Biochemistry.

Signature:
Name: Assist. Prof. Dr. Salman A. Ahmad
Date:

In view of the available recommendation, I found this thesis for debate by the examining committee.

Signature:
Name: Assist. Prof. Dr. Salman A. Ahmad
Head of chemistry Department
College of Science
Al-Nahrine University
Date:

Examination Committee's Certification

We the examining committee, certify that we read this thesis and have examined that student (Mariam Fadhil Nassir); in its contents and that, in our opinion; it is adequate as a thesis for degree of Master of Science in Biochemistry.

Chairman

Signature:
Name: Assist. Prof. Dr. Salman A. Ahmad
Date:

Signature:
Name: Assist.Prof.Dr.A.Amir A.Aziz Al-Suhail
Date:

signature:
Name: Assist.Prof. Dr.Salwa H. Al-Rubaei
Date:

Signature:
Name: Assist.Prof. Yihya Y. Zaki
Date:

Supervisor
Signature:
Name: Assist. Prof. Dr. Salman A. Ahmad
Date:

Approved for the college committee of Graduate studies

Signature:
Name: Assist. Prof. Dr. Laith Abdul Aziz Al-Ani
Address: Dean of the college of the Science Al-Nahrine University

الخلاصة

المقدمة:

يعتبرمرض الأكياس المائية من اهم الأمراض التي تعتبر كمشكلة مرضية وصحية في كثير من البلدان وخاصة العراق وفي الشرق الأوسط.

ويحدث هذا المرض نتيجة للدور اليرقي لدودةEchinococcus Granulosus و Echinococcus Multilocularis التي تستقر في الكبد أو الرئتين أو في باقي احشاء المضيف الوسطي , كالأبقار و الماعز والجمال.....الخ . يعتبر الأنسان كمضيف وسطي عرضي تحصل الأصابة لديه عن طريق تناول الخضراوات والمياه الملوثة ببيوض هذه الديدان أو عن طريق التماس المباشر مع الكلاب المصابة التي تعتبر المضيف النهائي لها. عندما ينضج الطفيلي يستقر في الأمعاء الداخلية للكلاب ويتحول الطفيلي الى كيس مائي. وتعتبر هذه المرحلة من المرض غير معدية ولاتظهر على المريض أي أعراض في البداية.

هدف الدراسة:

هناك بعض الدراسات التي توضح أن الأكياس المائية من صنف Echinococcus Granulosus أعتبرت من أهم المشاكل الصحية والشعبية بسبب عدم وجود دواء أو علاج ناجح لها.

تبين المواضيع التي ناقشناها في هذه الدراسة أن العلاج الناجح والفعال يكون في المراحل الأولية لنمو الكيس , لذلك فأن اهم مشكلة في تشخيص ومعالجة Hydatid Disease هي عدم وجود تحليل مختبري أو تشخيص يقدر على كشف المرض في المراحل الأولى للمرضى المصابين, ولذلك فأن الحاجة الى تحليل ثابت وملائم اصبح واجبا خاصة في المناطق التي ينتشر بها المرض.

العديد من الجهود قد اجريت في البلدان الغربية بصورة خاصة على صنف Echinococcus Multilocularis الى ان تم اكتشاف تحليل متوفر تجاريا وموثوق به لتشخيص هذا المرض وللأسف فأن هذا التحليل لم يستطع ان يكشف عن معظم المرضى المصابين بالأكياس من صنف Echinococcus Granulosusلذلك فان هذا التحليل لايمكن الأستفادة منه في البلدان التي يكثر بها ومن ضمنها بلدنا, لذلك اصبح من الضرورة دراسة كل مايتعلق ب Echinococcus Granulosus ,حيث اثبتت الدراسات المختبرية الكيميائية و العملية التي اجريت على انزيم الفوسفاتيز القاعدي ALP في جدار الأكياس المائية العقيمة والمخصبة الموجودة في كبد

المرضى المصابين أن أرتفاع فعالية انزيم ALPفي دم الأشخاص الذين يعانون أنسداد القناة الصفراوية بحصى المرارة يعتبرأو يعمل كبديل لصنف أنزيمALP في كبد الانسان. لذلك فأن الهدف من هذه الدراسة هو اظهار اختلاف انزيم ALP في جدار الأكياس المائية من النوعين المخصب والعقيم عن ALP في جسم الأنسان.

الطرق والمرضى:

ان هذه الدراسة قد اجريت على الأكياس المائية المستأصلة ودم الانسان.

تم تحصيل الأكياس المائية من كبد 6 مرضى مصابين ب Hydatid Disease من مستشفى الهلال الأحمر التخصصي في بغداد , حيث اجريت لهم عمليات الأستئصال الجراحية للأكياس مابين شهر كانون الثاني سنة 2007 ال شهر اب لسنة 2008 معتمدين في التشخيص على التصويربالموجات فوق الصوتية"السونار", اثنان منهم فقط هم من النوع المخصب والثلاثة الأخرى هي من النوع العقيم والرابع هو متحجر.

اما بالنسبة لعينات للدم فقد تم اخذها من المرضى المصابين بألتهاب القناة الصفراء وحصى المرارة, حيث تم اخذ المصل بعد فصله من الدم بعد ان تم اجراء تحليل نسبة انزيم -'5 nucleotidase حيث ان زيادة هذا الأنزيم تؤدي الى زيادة انزيم ALP في الكبد. العينات التي تم اخذها جمعت في وعاء واحد وتجانست وتم تقسيمها الى مجاميع حفظت في درجة حرارة -20°م الى ان يتم استعمالها.

تمت دراسة فعالية انزيمALP حسب طريقة Kind & King و تضمنت الدراسة تأثير العوامل المختلفة على الأنزيم كأختلاف تركيز المادة الأساس واختلاف pH ودرجات الحرارة, وثبات فعالية الانزيم عند تعريضه لدرجات حرارة مختلفة وكذلك عند استعمال -L phenylalanineكمثبط في كل من مصل الدم وجدار الأكياس المائية المنحل المتكسر والمتجانس.

النتائج:

1. تم تحصيل نتائج مختلفة عن فعالية إنزيم ALP في Hydatid cyst homogenated(HCH) من النوع المخصب الماخوذ من المرضى المصابين به بينما إنزيمALP ليس له فعالية في HCH في الأكياس العقيمة

2. من خلال نتائج الكشوفات الحياتية لأنزيم ALP نلاحظ ان قيم Km و pH ودرجة الحرارة التي يكون عندها الأنزيم اكثر فعالية مختلفة لكل منHCH و مصل الدم.
3. ان ميزة الحساسية للحرارة تبين ان انزيم ALP في صنف E. granulosus يكون اكثر مقاومة للحرارة من انزيم الكبد.
4. ان استجابة انزيم ALPلمثبط L- phenylalanine مختلفة في مصل الدم بينما لايوجد له أي تأثيريذكر فيHCH

الأستنتاج:

بالنظر لما تم اكتشافه في النتائج والمناقشة فهنا نوضح اهم الاستنتاجات التي توصل إليها

1. يعتبر انزيم E.granulosus ALP مؤشر على حياة الكيس المائي.
2. الخصائص المختلفة لأنزيم ALP في جدار الكيس المائي أظهرت أن أنزيم E.granulosus ALP مختلف بصورة جلية عنisoenzymes الأنسان وبصورة اكثر دقة عن ALP isoenzymes الكبد

السيرة الذاتية

الأسم : مريم فضيل ناصر شكرو الصائغ
تاريخ الميلاد : 1980/8/18
القومية: عراقية
رقم شهادة الجنسية : 320795
الحالة الاجتماعية: متزوجة
الجنس: أنثى
الديانة: مسيحية
هاتف: 07901195568
العنوان: العراق-بغداد/ الحبيبية/ حي 9 نيسان/ محلة 734/ زقاق 15/ دار 36
العنوان الالكتروني : Mary_fns@yahoo.com
الشهادة الحاصل عليها : بكالوريوس كلية العلوم/ قسم الكيمياء/ الجامعة المستنصرية
الوظيفة: معيدة
مكان العمل : كلية طب الكندي
تاريخ المناقشة: 2009/5/28
الأستاذ المشرف: أ.م.د.سلمان علي أحمد
أسم الأطروحة: مقارنة *التشخيص و التحاليل الحياتية لأنزيم الفوسفاتيز القاعدي في الأكياس المائية المتكونة من جنس Echinococcus Granulosus ومصل دم الأنسان*

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين/ كلية العلوم
قسم الكيمياء

مقارنة التشخيص و التحاليل الحياتية لأنزيم الفوسفاتيز القاعدي في الأكياس المائية المتكونة من جنس *Echinococcus Granulosus* ومصل دم الأنسان

رسالة مقدمة
الى
كلية العلوم جامعة النهرين
كأستكمال جزئي لمتطلبات نيل درجة ماجستير
علوم في الكيمياء الحياتية الطبية
من قبل
مريم فضيل ناصر
بكلوريوس علوم كيمياء (الجامعة المستنصرية 2002)

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2009 م