

ORIGINAL ARTICLE

Evaluation of Ameliorative activity of *Aloe vera* and *Bryophyllum pinnatum* extract on smokeless tobacco induced Hepato-toxicity in Albino mice

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ABSTRACT

The use of smokeless tobacco (ST) 'Gutkha' becomes a major public health challenge due to its strong potentiality for genotoxic and cytotoxic effects. Traditional plant based remedies in health care may be useful in this regard. The present study was aimed at evaluating the ameliorative effect of *Aloe vera* and *Bryophyllum pinnatum* leaf extract against smokeless tobacco gutkha induced toxicity in mice liver. The study was carried out in Swiss albino mice in which animals were treated with 100mg/kg and 300mg/kg bw doses of ST orally for 90 days. Other groups of mice were given ST along with *A. vera* and *B. pinnatum* leaf extracts individually and in combination (300 mg/kg bw) at an interval of 8 hours. Changes in liver were evaluated at gross morphological, histological and biochemical (enzyme SGOT, SGPT and ALP) level by following standard techniques. In ST treated groups, loss of body weight, increased liver weight and nodular hemorrhagic areas in liver surface were observed. Histologically, individualization and swelling of hepatocytes with fatty infiltration, coagulative necrosis as well as hyper chromatic and bizarre nuclear patterns had observed in liver of ST treated animals. These degenerative changes of hepatocytes were almost absent in the plant extract treated groups. Biochemically, a significant increase was found in liver enzymes activity in ST treated groups but it became significantly lower and at normal range in *A. vera* and *B. pinnatum* treated groups. Analysis of the result indicated ameliorative activity of *A. vera* and *B. pinnatum* leaf extracts against ST induced hepatotoxicity.

Keywords: Hepatotoxicity, *Aloe Vera*, *Bryophyllum pinnatum*, Ameliorative, Genotoxic, Cytotoxic

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INTRODUCTION

The use of smokeless tobacco (ST) product has been a common practice in India and other countries for many centuries. The chewing tobacco is mixed with areca nut, lime, catechu, some flavoring agents and is sold as legally commercial products termed as 'Gutkha'. The components of 'Gutkha' get mixed with saliva during chewing and the aqueous saliva extract is absorbed locally and through systemic circulation. The ingredients of smokeless tobacco are reported to possess cytotoxic, mutagenic and genotoxic properties which may lead to damage of the vital organs like liver, lung kidney etc [13]. Reactive oxygen species (ROS) are generated in the oral cavity during chewing which is favored by alkaline condition grown up by Ca(OH)₂ present in slaked lime [17]. This ROS is associated with highly reactive free radicals which can promote DNA damage, activate procarcinogens and alter the antioxidant defense mechanism of cell. Tobacco releases many Tobacco Specific Nitrosamines (TSN) in higher concentration, when kept in mouth. These TSN may undergo metabolic activation by cytochrome P450 and may lead to the formation of N-nitrosornicotine (NNK), which in further activation leads to DNA damage and cause cancer [17]. Studies reveal that, the chewing habit of ST products is associated with several harmful effects. Exposure to pan masala can cause serious toxic effect to embryo along with post-implantation loss, in-utero and lactational toxic effects [3]. ST products can also induce testicular damage, decrease sperm count and abnormal sperm head morphology [18]. Chronic feeding of Gutkha was shown to impair liver function in

mice with significant increase in Serum alkaline phosphatase (ALP), Glutamic oxaloacetic (GOT) and glutamic pyruvic (GPT) transaminases [31]. Increased cytogenetic damage was also reported in peripheral blood lymphocytes and exfoliated buccal mucosal cells of pan masala consumers [4]. It is also reported to be linked to the development of oral submucous fibrosis, a pre-malignant fibrotic condition [4]. Long term harmful effects of ST products have been established in the form of adenoma of liver, stomach and prostate; stomach papilloma, liver hepatoma, carcinoma and adenocarcinoma of lung and liver [18]. Smokeless tobacco Gutkha intake results in enhanced production and release of superoxide anion and thereby cause oxidative stress leading to cytotoxicity [36]. Several works have been carried out to evaluate the toxic potential of ST product Gutkha, however, there is paucity of literature regarding the amelioration of toxicity caused by ST through plant products.

Recent decades have shown a resurgent interest in plant based traditional remedies for many diseases. Due to presence of active compounds of various phytochemicals, plants are effectively used to treat numerous diseases. Plants also possess wide range of pharmacogenic property and less or no side effects unlike other synthetic drugs. Currently, research interest has been focused on plants having antioxidant and free radical scavenging activity, by virtue of which such plants may become effective remedy against different pathological conditions. Epidemiology shows that consumption of antioxidant rich plant products is beneficial to health, because it down regulates many degenerative processes and can effectively lower the incidence of some diseases and cancer [42]. Amidst the large variety of useful plants, *Aloe vera* (*Aloe barbadensis* Miller.) and *Bryophyllum pinnatum* are ethno-botanically important due to their role in health care and beautification. *Aloe vera* is a perennial plant belonging to the family Liliaceae, which is made of turgid green leaves joined at the stem in a rosette pattern [26]. Different phytochemicals present in *Aloe vera* are polysaccharides, Acemannan, anthraquinones, aloins, barbaloin, isobarbaloin, emodin, lipids, amino acids, enzymes and sterols [5,6]. It has a long history as medicinal plant with diverse therapeutic application due to presence of these important phytochemicals. Aloe gel has demonstrated wound healing, anti-inflammatory, antiviral, spermicidal and gastro protective properties [40]. It has also shown immune-stimulating and cholesterol lowering activity [8]. Several clinical trials also showed a blood glucose and lipid lowering effect for *A. vera* gel preparation [1].

Bryophyllum pinnatum, a perennial herb, belonging to family Crassulaceae, is used in folkloric medicine in many countries of the world. In traditional medicine, the leaves of this plant have been reported to possess antimicrobial, anti-fungal, anti-ulcer, anti-inflammatory, analgesic and antihypertensive activities [32,34]. A number of active compounds, including flavonoids, steroids, glucosides, bufadienolides and organic acids have been identified in *Bryophyllum pinnatum* [39]. The Methanolic extract of *B. pinnatum* leaves has reported to have histamine receptor (H1) antagonism in the ileum, peripheral vasculature and bronchial muscle [34].

Keeping in view, the beneficiary properties of *A. vera* and *B. pinnatum*, it is hypothesized that supplementation of these selected plant extracts individually or in combination may prevent the health hazards associated with smokeless tobacco Gutkha. The present work was aimed at studying the protective effect of *Aloe vera* and *Bryophyllum pinnatum* leaf extracts individually and in combination against smokeless tobacco induced toxic effects in liver of albino mice.

MATERIALS AND METHODS

Plant Extract preparation:

Aloe vera leaf gel extract was prepared by the method of Pawar *et. al.* (2005) with slight modification [39]. Mature, healthy and fresh leaves of *A. vera* were collected, authenticated and washed with fresh water. The thick epidermis was selectively removed. The solid leaf gel was dried in the oven at 80°C for 48 hours and then grinded to powdered form by using Mortar and Pestle. 20 grams of this powder was soaked in 200 ml. of methanol for 24 hours with occasional shaking. The contents were then filtered through Whatman filter paper no. 1 and the filtrate was evaporated to dryness. This dried extract was further powdered and then dissolved in distilled water. Freshly collected, mature leaves of *Bryophyllum pinnatum* were washed and dried. About 10 grams of dried powdered leaves of *B. pinnatum* was uniformly packed into a thimble and extracted in Soxhlet extractor with 260 ml of methanol. The extraction process was continued for 24 hours (6-7 cycles). After that the extract was taken in a beaker and kept on hot plate and heated at 30-40°C till all the solvent got evaporated. Dried extract was further powdered and mixed with distilled water [14].

Phytochemical Screening: Preliminary phytochemical screening of Methanolic extract of *A. vera* and *B. pinnatum* was carried out to study the presence of bioactive compounds by following standard techniques [10,41,43].

Animal Maintenance and experimental design:

Swiss albino mice weighing 20-25 g were obtained from King Edward Pasteur Institute, Shillong (Meghalaya), India. The animals were maintained in standard environmental conditions (24±2°C temperature and 12 hours light/dark cycle) throughout the experimental period. The animals had access to commercial pellet diet and water *ad libitum*. All institutional and national guidelines for the care and use of laboratory animals were followed (CPCSEA guidelines-Committee for the purpose of control and supervision on experiments of animal, India). Animals were randomly divided into six groups (of 5 mice each) :

Group A: Control (feed on formulated diet),

Group B: ST-1 (Male Mice were orally administered ST in dose level 100 mg/kg b. wt)

Group C- ST (Male Mice were orally administered ST in dose level 300 mg/kg b. wt)

Group D- ST AV (Male mice were orally administered ST 300 mg/kg b.wt ; beside 300 mg/kg b. wt. *Aloe vera* extract at an interval of 8 hours)

Group E- STBP (Male mice were orally fed ST 300 mg/kg b.wt ; beside 300 mg/kg b. wt. *B. pinnatum* extract at an interval of 8 hours)

Group F- STC (Male mice were fed orally ST 300 mg/kg b.wt ; beside leaf extract of *A. vera* and *B. pinnatum* in combination in dose level 300 mg/kg b. wt mixed in 1:1 proportion at an interval of 8 hours)

Normal diet was given twice daily, individually and the residual was measured next day. Animals were sacrificed after 90 days .During the observation period, all animals were regularly weighed and any abnormal signs and symptoms were recorded.

Histopathology:

The animals were sacrificed at 30, 60 and 90 days by cervical dislocation under anaesthesia, and the liver was excised for gross morphological and histological study. Tissues were fixed in Carnoy's fixative, dehydrated through graded ethanol series and embedded in paraffin wax by following standard procedure. About 4µm thick sections of tissue were stained with Eosin and Haematoxyline method, Luna 1964 [25] and microphotographs were taken in microscope.

Biochemical assays:

The blood was collected by cardiac puncture and serum was separated by centrifugation for estimation of biochemical parameters –SGOT SGPT and ALP for liver functional observation. Serum GOT and GPT enzyme activities were studied by following standard method of Reitmen and Frankel, 1954 with modification. ALP activity was studied by the method of King and Armstrong, 1954 [16,38].

RESULTS

Phytochemical Screening:

Preliminary phytochemical screening revealed the presence of some active compounds viz. Carbohydrate, amino acids, flavonoids, tannin, phenolic compounds and vitamins in both *A. vera* and *B. pinnatum* extract. In addition to these, *B. pinnatum* leaf extract also contain saponin, glycosides and caratenoid compounds, details of which are given in Table 1.

Gross morphological alterations:

The experimental animals showed signs of intoxication such as loss of fur, rough skin, loss in body weight and food intake capacity during the period of exposure of ST. However, the mice of *A. vera* and *B. pinnatum* treated groups (group D, E and F) showed improvement in weight and skin structure. The data on body weight for control and treated groups are represented in **fig 1**. The ST groups of mice showed a decreased body weight and increased liver weight compared to control animals. Whereas the other treated group animals did not showed such decline in body weight. Grossly, small lesions and nodular structures with hemorrhagic areas were found to develop on the surface of liver in ST-1 and ST group. Such structures were not seen among the animals of STAV, STBP and STC group.

Histopathological Alteration:

Histological studies of liver section of ST and ST-1 group animals revealed severe alteration in comparison to control group and *A. vera* and *B. pinnatum* treatment group. Individualization of hepatocytes with loss of intercellular cementing material, presence of numerous mitotic figures and hepatocytes with karyorrhectic and karyolytic nucleus were observed in liver sections of mice from ST-1 group (fig 2-B,C). However, in ST treated group animals, cytolysis and bizarre nuclear pattern with shrunken chromatin material had observed. Fatty changes and presence of fibrinous thrombus with infiltrated leucocytes within the central vein had also observed in ST treatment group (fig 2-D, E, F). All these changes were not developed in STAV, STBP and STC treatment group. The sections from STC treated animals showed minimum cytolysis and simultaneous regeneration activity of disturbed liver

architecture (fig 2-G,H). The liver sections from control group showed normal arrangement of hepatocytes (fig 2-A).

Biochemical parameters:

The effect of treatment of Smokeless Tobacco product gutkha(ST-1 and ST) and co-administration of ST with *A. vera* and *B. pinnatum* (STAV, STBP and STC) extract on Serum Glutamate Pyruvate Transaminase(SGPT) , serum Glutamate Oxaloacetate Transaminase(SGOT) and serum alkaline phosphatase levels(ALP) are shown in table 2, 3 and 4. Treatment of ST-1 and ST in swiss mice resulted in significant elevation of SGOT, SGPT and ALP levels in comparison to the control group and plant extract treated group animals. The enzymatic activity increased with the increase in treatment period in ST treated animals. On the other hand, the enzymatic activity was decreased in STC group animals.

DISCUSSION

The smokeless tobacco 'Gutkha' is a mixture of various components which possess mutagenic and genotoxic properties as shown in several short term assays [12]. The habit of consuming Smokeless tobacco (ST) product is increasing due to ease of availability, cheap cost and increasing popularity by advertising agencies. This product is responsible for various deleterious effects on different organs of animal as well as human being. It has been estimated that about 5 million young Indians are suffering from Oral Submucous Fibrosis (a disease which is precursor of oral cancer) as a result of increased popularity of habits of chewing smokeless tobacco product Gutkha and pan masala [29]. These products are also responsible for increased cytogenetic damage in peripheral blood lymphocytes and exfoliated buccal mucosal cells of habitual chewer [4]. The users often swallow the liquid extract of Gutkha and other smokeless tobacco products which increases the possibility of carcinogenic effects at sites other than only oral cavity.

The long term bioassay is generally carried out with an aim to simulate the human situation in the laboratory animals to focus on the toxicity produced by any harmful agent. The result of the present work indicates that smokeless tobacco 'Gutkha' has toxic effect on mouse liver in vivo. Human subject can also develop certain liver damages as a result of consumption of ST product Gutkha. In this study, the mice treated with smokeless tobacco (ST) showed reduction in their body weight as compared to control and *A. vera* and *B. pinnatum* treated groups. These changes may be related to chronic nutritional insufficiency and possibly to liver toxicity, reported to occur after long term pan masala consumption [27]. The mice treated with 100 mg/kg and 300 mg/kg b wt. for 90 days showed prominent structural changes in liver at gross and histological level. An increased trend of liver weight had observed in ST-1 and ST group of animals. The presence of nodular structure with hemorrhagic areas at the liver surface in ST-1 and ST treated groups indicates probable toxicity in that vital organ. The liver histology of ST-1 (100mg/kg b wt) treated animals showed loss of intercellular cementing material, presence of mitotic figures and hepatocytes with karyorrhectic and karyolytic nucleus. Certain hepatocytes showed fragmented nucleus and other showed complete dissolution of the nucleus (karyolysis). In ST treated animals cytolysis and bizarre nuclear pattern had observed. Hepatocytes of this group also showed generalized fatty infiltration. Usually, micro-vesicular fatty changes are seen as myriads of tiny fatty droplets which surround the centrally located nucleus. Here, certain fat droplets were seen as comparatively large round empty vacuoles which displace the hepatocytes nucleus to the peripheral areas of cell. The liver cells also indicated the presence of fibrinous thrombus with infiltrated leucocytes within the central vein. Similar alterations were also observed by Nigam and Bhatt (2012) [31] which stated that severe fatty changes as a result of pan masala exposure may impair cellular function and can cause irreversible damage to some intracellular process such as decrease of protein synthesis. Nigam and Bhatt in 2011 also reported about endogenous initiation of hepato-carcinogenesis in rodents, which occurs as a result of intracellular production of H₂O₂ that causes DNA strand breakage as well as chemical alterations of DNA bases [31]. Generally, such damage to DNA initiates biochemical alterations that lead to neoplastic transformation and cancer. In the present study, severe alterations of liver had observed in case of both ST treated group. However, such alterations were not observed in STAV, STBP and STC treated groups. Even, it can be considered as amelioration activity of *A. vera* and *B. pinnatum* both individually and in combination.

There is direct relationship between the enzymatic activity and the level of damage or degree of protection to any organ. Significant increase in enzymatic parameters demonstrates excessive damage to the body organs. The present study focuses on the level of damage or protection of Liver, selecting certain marker enzymes of liver. Serum Glutamate Pyruvate Transaminase, serum Glutamate Oxaloacetate Transaminase and serum alkaline phosphatase are extremely used as marker enzymes. The present study indicated that Smokeless tobacco Gutkha caused a significant increase in the aforesaid enzyme activities

as compared to control and STC treated animals. Increased level of ALP can be correlated with cellular injury, inflammation, tissue damage and progression of fibrosis in chronic disorder of certain organs like lung [19]. Significant increase in tissue ALP activity after administration of ST product Gutkha may be due to increased functional activity of various organs, which probably leads to *de novo* synthesis of the enzyme molecules as suggested by Yakubu et al, 2005 [44]. In the present study, a higher level of serum GOT and GPT was observed in both ST treated groups of mice in comparison to control and *A. vera* and *B. pinnatum* treated mice. Such rise in GOT and GPT activity is a sensitive indicator of damage to the cytoplasmic and mitochondrial membrane due to liver toxicity [30]. GOT and GPT are commonly measured transaminases, which can leak out into the blood stream due to increased permeability of cell membrane of hepatocytes as a result of severe damage to liver. Cellular membrane damage or necrosis may cause release of these enzymes into blood. Studies revealed that nicotine of tobacco can cause destruction of hepatic cell membrane which in turn releases cytosomal enzymes of hepatocytes resulting into increasing serum level.

The toxic potential of Smokeless tobacco product is due to its contents, i.e., areca nut (arecoline), catechu, polyaromatic hydrocarbons, nitrosamine, toxic metels (Pb, Cd, Ni), residual pesticides (DDT, BHC and their isomers) and fungi (*Aspergillus* Species) which promotes carcinogenesis by reacting with cysteine in vivo and in vitro. As a result of this reaction, cystein/3-alkylation adduct is formed that leads to carcinogenesis [37]. Also, the released superoxide ions can initiate carcinogenesis of many organs by way of autoxidation of polyphenols and interaction of catechin(tannin) with lime. Mukharjee et al. in 1991 showed that administration of pan masala to swiss albino mice in the dose range of 8-200 mg/kg body weight was found to cause cytogenetic damage in bone marrow cells and sperm head abnormality [27]. The toxic substances present in smokeless tobacco products can accumulate in the body and the food chain and thereby, can pass through liver and kidney during metabolic processes. It seems that during nicotine metabolism, free radicals are produced that can trigger lipid peroxidation and reaction with DNA and membrane proteins, thereby causing cell damage [7]. Plant based antioxidant compounds can produce inhibitory effects on cytochrome P450 and prevent nicotine metabolism which can reduce production of free radicals. *Aloe vera* and *Bryophyllum pinnatum* are two perennial herbs with several beneficiary properties. *Aloe vera* contains a number of beneficiary chemicals including antioxidants that may exert useful effect on tobacco mediated toxicity. More than 75 ingredients have been identified from the *Aloe vera* gel, each of which may have a range of mechanism of actions. Besides these, it contains at least seven super oxide dismutases with antioxidant activity and can exert a protective effect in oxidative stress conditions [23]. Likewise, *Bryophyllum pinnatum* also contains a wide spectra of chemicals due to which it may have a high efficacy towards smokeless tobacco induced toxic stress. *B. pinnatum* has been found to inhibit membrane lipid peroxidation and having anticarcinogenic, antioxidant and anti-inflammatory properties [11,22]. The present investigation demonstrates that smokeless tobacco has the potential to cause damage to liver tissue. Some of these damage can be improved by administration of *Aloe vera* and *Bryophyllum pinnatum* extract individually or in a combined way. Therefore, *Aloe vera* and *Bryophyllum pinnatum* both could be useful to protect the liver from smokeless tobacco induced toxicity.

Table-1-Phytochemicals present in *Aloe vera* and *Bryophyllum pinnatum* leaf extract after initial screening

Chemical compound present	<i>Aloe vera</i>	<i>Bryophyllum pinnatum</i>
Carbohydrates	+	+
Proteins and amino acid	+	+
Phenols and Tannins	+	+
Flavonoids	+	+
Saponin	-	+
Glycosides	-	+
Carotenoids	-	+
Vitamins	+	+

Table-2: Level of Serum Glutamate Oxaloacetate Transaminase in control and treated group albino mice (values are mean±S.E. of 5 animals)

Treatment Period (Days)	GOT(IU/L)					
	Control	ST-1	ST	STAV	STBP	STC
30	76.4±0.57	91.3±0.09	132.1±0.19	81.4±1.27	87.8±0.17	78.9±0.43
60	79.8±0.03	111.9±0.12	196.5±0.21	117.8±0.83	104.9±0.22	95.5±1.37
90	77.1±1.08	208.7±0.31	275.7±1.16	142.1±1.31	147.4±0.31	122.0±1.03

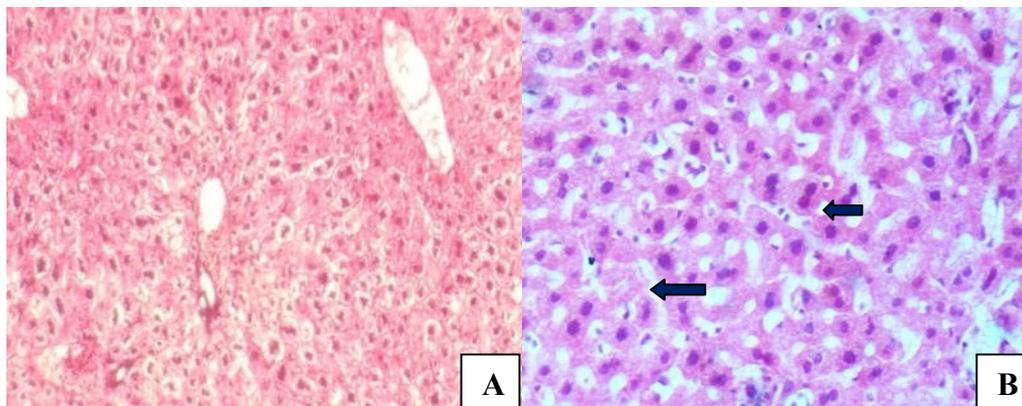
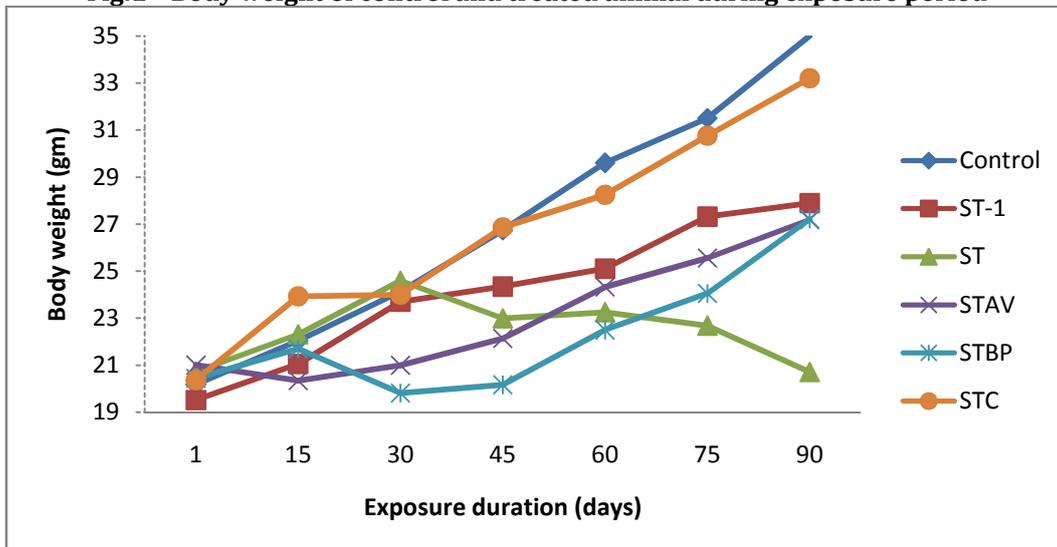
Table-3: Level of Serum Glutamate Pyruvate Transaminase in control and treated groups of albino mice (values are mean±S.E. of 5 animals)

Treatment Period (Days)	GPT(IU/L)					
	Control	ST-1	ST	STAV	STBP	STC
30	30.6±1.01	36.1±0.32	73.7±0.17	35.3±1.06	33.4±0.68	42.2±0.08
60	32.5±0.12	96.1±0.02	121.4±1.15	54.0±1.23	62.1±1.07	74.5±0.61
90	31.1±0.23	113.8±0.04	147.1±0.54	96.1±1.11	103.8±0.91	86.3±0.20

Table-4: Level of Serum Alkaline Phosphatase (IU/L) in control and treated groups of albino mice (values are mean±S.E. of 5 animals)

Treatment Period (Days)	ALP (IU/L)					
	Control	ST-1	ST	STAV	STBP	STC
30	52.9±0.11	48.5±0.14	61.1±0.41	51.8±0.08	46.8±0.04	50.6±0.15
60	54.2±0.05	79.22±0.36	167.8±0.11	70.6±0.16	59.4±0.12	74.1±0.06
90	54.9±0.13	134.2±0.34	216.3±0.16	88.6±0.31	71.7±0.16	67.9±0.09

Fig:1-- Body weight of control and treated animal during exposure period



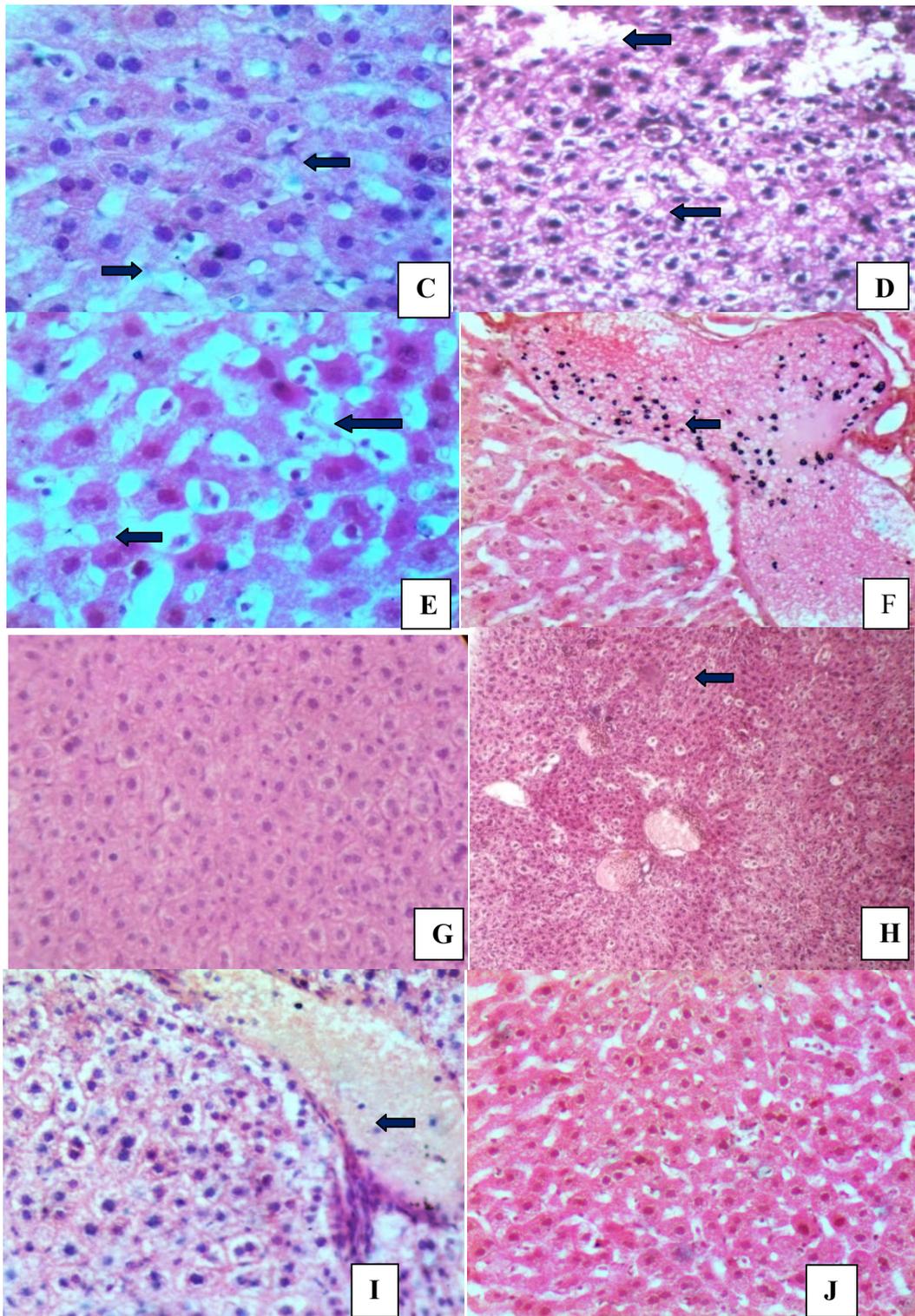


Fig:2- Microphotograph of histological study of mice liver :-A-Control group liver showing normal histological structure (x200); B- Karyorrhactic and karyolytic nucleus of hepatocytes with mitotic figures after 60 days ST-1 treatment(x200); C- Hepatocytes losses intercellular cementing material with shrunken chromatin after 90 days ST -1 treatment (x400) , D- Cytolysis and fatty changes of hepatocytes with bizarre nuclear pattern after 30 days of ST treatment (x200) ; E- Liver histology after ST treatment of 60 days showing cytolysis and fatty changes with bizzare nucleus(x400) ; F-Presence of Fibrinous thrombus with infiltrated leucocytes within the central vein after 90 days of ST treatment (x200), G- liver section from STC group after 60 days of treatment showing resemblance to normal liver architecture (x200); H- Liver after 90 days of STC treatment shows regeneration of hepatocytes leading to normalized structure (x100), I- Liver structure after 90 days of STAV treatment showing minimum leucocyte infiltration in central vein (x200), J- Liver after 60 days of STBP treatment (x200)

CONCLUSION

The present study indicates the toxic potential of smokeless tobacco 'gutkha', which is now, become widely prevalent among the all age group people of our society. Despite the well evidence of health hazards caused by these products, its uses have been increasing in a growing trend. The present work investigated the range of toxic stress exerted by this smokeless tobacco product on mice liver. Simultaneously, an evaluation was made to reduce or mitigate the toxicity caused by ST product by using *Aloe vera* and *Bryophyllum pinnatum* extract singly and as a mixture of equal amount. An encouraging result was found that administration of this *A. vera* and *B. pinnatum* individually or in combination can effectively combat the harmful effect of Smokeless tobacco Gutkha. Various beneficiary activity including antioxidant activity of this two plant may be the major reason for its positive effect on liver parameter. However, further investigation is required to define the proper reaction mechanism of these plants.

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