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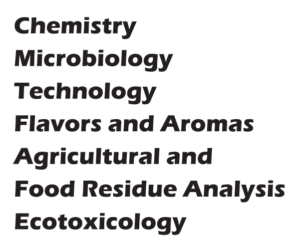

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ANTICANCER ACTIVITY OF *ASPERGILLUSORYZAE-VAR.EFFUSES*KOJIC ACID IN VITRO AND IN VIVO USING RATS

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ABSTRACT

Kojic acid (5-hydroxy-2-hydroxymethyl-1,4-pyrone) used in the present study was obtained from *Aspergillus oryzae* var. *effuses* cultures.Cytotoxic activity of kojic acid was examined in vitro using colon (HCT 116), liver (HEPG2), cervical (HELA) and brest (MCF7) carcinoma cell lines. Kojic acidshowed a high percentage cell death of HCT-116 and HEPG2 carcinoma cell lines than the other two cell lines (MCF7 and HELA), indicating promising anti-tumor properties and demonstrates their effect on colon and liver cancers cell proliferation.A marked reduction were observed in the levels of ALP (45% and42%), ALT (45% and 44%) and AST (34% and 35%)in sera of rat groups given kojic acid(100mg/kg) compared to 1,2 dimethyl hydrazine (DMH)and diethylnitrosamine (DEN) control rat groups (C1 and C2 respectively) indicating improvement effect of Kojic acid. Highest significant decreases were observed in the levels of γ-GT (48% and 54%) in sera of kojic acid rat groups. Anticancer activity was evaluation through determination of CEA and C19.9 in chemically-induced cancer rat groups treated with kojic acidcompared DMH and DENcontrol rat groups (C1 and C2). Thereforekojic acid is more effective for inhibiting DMH and DEN induced colon and liver cancers. The present results showed the activity The most significant findings of the present study is that the kojic acid (200 mg/kg body weight) have shown beneficial effect on DMH and DEN - induced groups. According to these observations, the use of kojic acid can be recommended as anticancer agent.

KEYWORDS:
Kojic acid, Anticancer, Cytotoxicity, DMH, DEN, Rat.

INTRODUCTION

A fungus is a member of a large group of eukaryotic organisms that include microorganisms such as yeasts and molds, as well as the more familiar mushrooms. These organisms are classified as a kingdom of Fungi, which are separate from plants, animals, and bacteria. The fungus biomass containing different metaboliteshave various uses as food or feed [1,2,3],biopharmaceuticals and treat various human diseases[4,5,6]. Different metabolites such as ethanol [7], citric acid [8], enzyme[9], polysaccharides [10] have been produced from fungus.Kojic acid (5-hydroxy-2-hydroxymethyl-1,4-pyrone) is a major secondary active fungal metabolite produced by a variety of fungus, including *Aspergillus oryzae, Aspergillus flavus*, and *Aspergillus tamarii*, as well as *Penicillium* species and certain bacteria [11,12]. Ibrahim [13] producedhigh yield of kojic acid crystals by Aspergillus oryzae var. effuses.Kojic acid iswidely used in food industries, as a food[3] depigmenting agent in the cosmetic industry[2,14]. Kojic acid was used in medical as anti-bacterial, antifungal and antineoplastic activities [3,14,15]. Kojic acid is also used as an important material in antibiotic [16]. Several investigators studied the antioxidant and anticancer activities of Kojic acid [15,17].Moreover, kojic acid as iron chelator seem to be safe and their toxicity as food, drug or cosmetic agents with usual therapeutic doses have not been reported yet [18,19].

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries [20]. Colonand hepatocellular carcinoma are the most malignant tumors with very high morbidity and mortality rates.Colon and liver carcinomas are the most common cancers accounts highest percentage of cancer [21,22]and the third leading cause of cancer-related mortality worldwide [23,24].However, the therapeutic approaches for colon and livercancers are still very limited and expensive[22,25,26]. The prevention and treatment of colon and hepatic cancersare associated with diet[27,28]. In this regard, fungal metabolites could be used to reduce the risk and inhibit the development of cancers considering the continuing need for the improvement of therapeutic activity and new effective anticancer agents with no toxic side effect[2,29]. Chemotherapy is one of the most frequently used therapeutic modalities for

treatment of cancer [2,28].Fungal metabolites are a rich resource of cancer chemotherapy drugs and target tumor cells[25,26]. Experimental studies demonstrated that many microbial metabolites have anticancer potential in a variety of biological assays and animal models[30,31,32], therefore, the increase interest in the identification of various fungal metabolites for developing cancer therapeutics [15,25,33]. In recent years, different metabolites are isolatedfrom some microorganisms as bacteria, fungi and algae showedhealth effectand they are anti-canceriious, antimicrobial and anti-inflammatory[34,35,36,37].Microbial metabolites produced from microorganisms have been successfully tested in animals and humans with anticarcinogenic [36,37]. Mushroom metabolites have been isolated [38] to prevent tumor growth in rat and mice [39,40,41]. Other investigators provided evidence the microbial metabolites consumption results in protection against chemically induced large bowel cancer [31,42,43].

However, the antitumor activities of the produced fungal metabolites were evaluated in an in vitro study, but little research was published on their antitumor activity in vivo. In view of this,the present study focusing on anticancer effectof kojic acid produced from *Aspergillus oryzae* var. *effuses* culturesin vitro and on chemically induced colon and liver cancers in vivo using male albino rats.Anticancer activity was evaluation through determination of biochemical parameters andhistological examination of liver and colon sections of rats..

MATERIALS AND METHODS

Carcinogenic materials used in this study were, 1, 2 dimethylhydrazine dihydrochloride (DMH) and diethylnitrosamine (DEN)were obtained from Sigma-Aldrich® chemie, Gmbh, Riedstr. 2, D-89555 Steinheim, Germany. Freshly diluted DMH and DEN in saline to a final concentration were used.

Kojic acidpurified crystals wereobtained from *Aspergillus oryzae var.* effuses cultures[13] and ground in a food grinder to a very fine powder and stored till used.

In vitro studies. Cytotoxicity test of kojic acid was done in vitro usingdifferent human carcinoma cell line particularly thoseof colon (HCT 116),liver (HEPG2), brest (MCF7) and cervical (HELA) carcinoma cell linesObtained from National Cancer Institute, Cairo University. Measurement of potential cytotoxicity of kojic acid was assayed according to the method described by Skehan et al. [44]and Jwanny et al. [31].

In vivo studies. Induction of colon cancer experimentally in rats was done using1,2dimethyl hydrazine [45] and diethylnitrosamine (DEN)was used for liver cancer [46,47].

Animals. Twenty eight adult male albino rats (12 weeks of age, weighingabout 120 -130g) were purchased from the National ResearchCenter for biological products. The rats were divided into fourgroups (7 rats/group) and housed in a wire screen cage. The ratshad free access to fed commercial diet and tap water. The animalroom was controlled (25 ± 1ºC) and had a 12-hour light-darkcycle and humidity at 60 ± 5%. The rats were allowed to acclimatize for two weeks before the experiments began. Tworat groups werechemically induced colon cancer using subcutaneous injections of 1,2-dimethyl-hydrazine (DMH) at a dose of 40 mg/kg body weight for 5 weeks (twice/week)according to the method described previously [45]. A group of the administrated DMH rat was maintained without any treatmentover experimental period (20weeks) and used as colon carcinogeniccontrol rats (C1). Other administrated DMH rat group was treated withoral dose (100 mg/kg body weight/day) of kojic acid(C1/T) till the end of theexperimental period (20 weeks). Other two rat groups were chemically induced liver cancer using subcutaneous injections of diethylnitrosamine (DEN)at a dose of 40 mg/kg body weight for 5 weeks (twice/week) according to the method described previously [46,47].A group of the administrated DEN rat was maintained without any treatment over experimental period (20weeks) and used as liver carcinogenic control group (C2). Other administrated DEN rat group was treated with oral dose (100 mg/kg body weight/day) of kojic acid (C2/T) till the end of the experimental period (20weeks). The experimental protocol was done according tothe methods of Moharibet al. [10].

Sample preparation. At the end of experimental period (20 weeks), blood samples were drawnfrom 7 ratsper each group separately using capillary tubes, centrifuged at4000 xg for 10 min. Separated sera or plasma were used for differentbiochemical analysis. Liver and colon were removed and used forpathological examinations.

Biochemical parameters. Total protein (gm/dL) was estimated [48]using Biodignostic kits, Egypt. Serum albumin level was measured according to the method previously described [49]. Globulin was calculated by subtracting albumin form the total protein [50]. Alkaline phosphatase (ALP) level (IU/L) was carried outreferring the DGKC indications [51]. Aspartateaminotransferase (AST) and alanine aminotransferase (ALT)levels were measured (U/L) according to the method of Reitmanand

Frankel [52], using kits of QCA, Spain. Gamma glutamyltransferase (γ-GT) was carried out according to the kineticcolorimetric method [53], using Biodignostic kits,Egypt. Quantitativedetermination of CEA (ng/ml) was performed with commerciallyavailable Enzyme Immunoassay Kit[54], Bio Check, Inc. catalog number: BC-1011. CA 19.9 was performed[55] with commercially available EnzymeImmunoassay Kit (Invitrogen, catalog number: 99-0070).

Statistical Analysis. All statements of significance were based on aprobability of $P < 0.05$. Data from the molecular biology studieswere analyzed using the General Liner Models (GLM) procedureof Statistical Analysis System [56] followed by theScheffé-test to assess significant differences among groups.

Histology. Histological assessments of colon and liver tissues werecarried out usingHematoxyline and Eosin (H&E) staining technique [57].

RESULTS AND DISCUSSION

In recent years, much attention has been focused on some metabolites isolated as natural sources from bacteria, fungi, algae and plants. Their wide range of biological activities and toxicity are the main reasons for the increase interest towards themby several investigators [10,31,34,58].

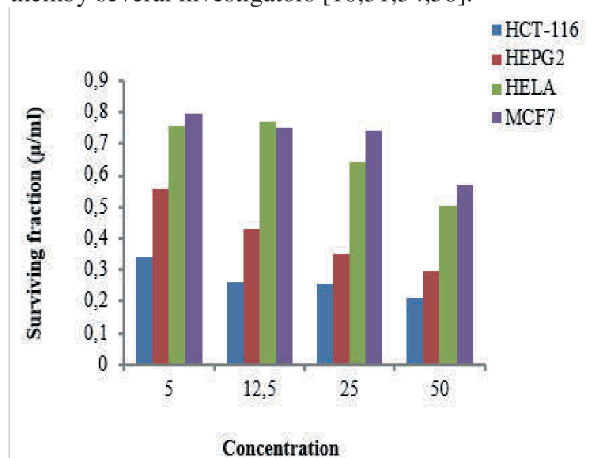

FIGURE 1
Cytotoxicity effect of kojic acid on carcinoma cell lines.

In vitro studies. Measurement of potential cytotoxicity. Kojic acid was obtained previously from *Aspergillus oryzae var.* effuses cultures [13]. The main objective of this study was to evaluate the potential efficacy of the obtainedkojic acid against colon and liver cancers in vitro and in vivo.The present study was carried out to screen the purified kojic acid using in vitro cytotoxicity test to identify the activity of kojic acid in growth inhibition of different tumor cell lines. Cytotoxic activity of kojic acid was examined using liver (HEPG2), colon (HCT 116), breast (MCF7) and cervical (HELA) cancer cells in vitro. The results indicated that kojic acid has anticancer effect against all carcinoma cell line, particularly colon and liver carcinoma.Results (Figure 1) showed that kojic acid was more effective in inhibition of HCT-116 and HEPG2 but lower effective against HELA) and MCF7human cancer cells in vitro. Kojic acid exhibited more effectiveness on HCT116 but less effect on HEPG2human cancer cells.Similar results were reportedin different studies in vitro by other investigators [1,3,22,33,41] when examined different fungal products on different human cancer cell lines in vitro. So it can be observed that kojic acid,as active fungal product, inhibit cell proliferation ofhuman colon cancer cell line (HCT-116) than that ofhuman livercancer cell line (HEPG2). The kojic acid anti-proliferative effect in vitro couldbe explains the arrest cell cycle and generate apoptosis [35]. Results in Figure (2), illustrate the dose response (IC50)of kojic acid on HCT116 and HEPG2 cells. The present results also, showedthe growth inhibitory effect of kojic acid on HCT116 and HEPG2human cancer cell lines.This indicated that kojic acid have anticancer activityagainst colon and liver carcinoma. The kojic acid reduced the survivalfraction to 50% (kills 50% of the cancer cells) particularly HCT116and HEPG2cancer cell lines (IC50).The present resultsindicated that thekojic acid has appreciable anti-cancer activity on HCT116 greater thanthat of HEPG2.However, the different effects of fungal metabolites are dependants on their type (dose, structure, soluble and insoluble) and on the duration of the experiment[10,31].

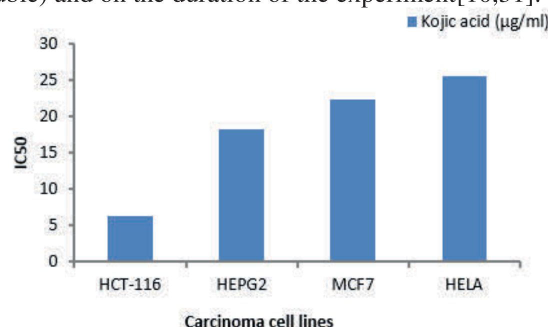

FIGURE 2
IC50 of kojic acid on different human cancer cell line.

In vivo studies. Kojic acid is widely used as food and in food industries [59], their toxicity have not been reported as food, drug or cosmetic agents with therapeutic doses [19]. Kojic acid was found to be poses no risk of genotoxicity or toxicity in rats and in mice [60]. Moreover, acute toxicity resulting

from oral kojic acid dose has never been reported [5]. Results in Figure (1) showed that kojic acid was more effective in inhibition of HCT-116 and HEPG2 but lower effective against HELA and MCF7human cancer cells in vitro.The present study was done in order to investigate the anticancer activity of kojic acid on the chemically induced colon (HCT116) and liver (HEPG2) cancersin vivo usingmale albino rats. The colon cancer was induced by intra-peritoneal injection of 1,2-dimethyl-hydrazine (DMH) at a dose of 40 mg/kg body weight (twice a week for 5 weeks). Previous studies indicated that theDMH treatment led to the development of colon carcinoma [10,45].Other investigators [31,43,45] reported DMH at a dose of 40 mg/kg body weight was sufficient to the development of colon cancer.The liver cancer was induced by intra-peritoneal injection of diethylnitrosamine (DEN) at a dose of 40 mg/kg body weight (twice a week for 5 weeks). Previous studies indicated that DEN treatment led to the development of hepatocellular carcinoma [46,47,60]. Several workers [21,27,33] enhanced DEN and studied the inhibition of two stage renal carcinogenesis.However, following DMH and DEN exposure, there was a marked increase in the incidence of colon and liver cancers [5,15,21,61].Kojic acid was not clastogenic in a comet assay in the liver, stomach and colon indicating that Kojic acid probably is not a germ cell mutagen [62]. Moreover, some investigators [14,15,18, 60] reported that the kojic acid is a non-genotoxic carcinogen after investigated the rat and mice fed kojic acid containing diet for a long period (12-78 weeks). In the present study orally dose (100mg/kg b.w) of kojic acid was usedin treatments of DMH and DEN induced colon and liver cancers respectively. Results indicated that the kojic acid dose (100mg/kg b.w.) was sufficient for treatment of both chemically induced colon and liver cancers.These results are in accordance with those reported by other investigators [4,15,22,60] when they examined different doses of different products in humans and animals.

Biochemistry. In the present study, The results in table (1) show that, the alkaline phosphatase (ALP), alanine aminotransferase enzyme (ALT), aspartate aminotransferase enzyme (AST) and Gamma glutamyl transferase (γ-GT) levels were significantly higher in rat groups (C1 and C2) received the carcinogenic materials (DMH and DEN) more than those in rats received the carcinogenic material for 5 weeks then treated with the kojic acid (C1/T and C2/T). Serum transaminases are considered to be sensitive indicators of liver injury. In DMH-induced cancer rats, the liver was necrotized. Cheng et al. [45] reported that the dose of DMH was sufficient to induce colon cancer in rats. Tsujiuchi et al. [46]and Shirakami et al. [47] reported that the dose of DEN was sufficient to induce liver cancer development in rats. The hepatic damage was indicated by increases in ALP, ALT and AST levels.ALP, ALT, AST are reliable markers of liver function [33,63]. Liverdamage induced by chronic treatment that leads to liver cell necrosis and consequentlyelevated levels of serum transaminases. Theseincreases of transaminases by carcinogenic materials are consistentwith previous reports [39].The increased serum ALP, ALT, AST levels were reported in cancers and it may be due to liver dysfunction [63,64]. An increase in the ALT, AST levels in plasma might be mainly due to the leakage of these enzymes from the liver into the blood stream which gives an indication of the hepatotoxic effect [65,66]. However, the ALP, ALT, AST levels were significantly elevated in DMH and DEN carcinogenic rats and consistentwith other previous reports [67]. The enhancement of prooxidant effect of 7,12-dimethylbenzen in rat was studied previously[68], and found the alteration of liver function was revealed by the increase in marker enzymes (AST, ALT, ALP) than their control. Other investigators reported the increase intransaminases in rats are due to increase in proteinsynthesis in the rat liver[66,67]. From the present data, it can be observed that, there is difference between C1 and C2 groups in the values of ALP, ALT and AST activities [21,30].

TABLE 1
Biochemical parameters in sera of experimental rat groups.

Parameters	C1	C1/T	C2	C2/T
ALP (IU/L)	254.00±7.20	140.00±4.80	230.00±8.20	134.00±5.20
ALT(U/ml)	72.00±5.20	40.00±1.00	58.00±3.80	32.00±1.80
AST(U/ml)	82.00±4.20	54.00±0.80	74.00±6.20	48.00±1.40
γ-GT (U/L)	120.00±6.40	62.00±3.10	160.00±6.40	74.00±2.60
Total protein (g/dl)	6.00±0.40	6.60±0.40	5.20±0.40	6.00±0.20
Albumin (g/dl)	4.00±0.40	4.20±0.40	3.40±0.40	3.80±0.20
Globulin (g/dl)	2.20±0.20	2.40±0.20	1.80±0.10	2.20±0.20

Data was presented as mean value ± SE (7 rats / group).

Generally, results of the present study showed higher significant decreases in the levels of ALP, ALT, γ-GT and AST in sera of rats of administered kojic acid (100mg/kg b.w.) as compared to those administered DMH and DEN (C1 and C2 control rat groups respectively). Results in table (1) showed higher significant decreases in the levels of ALP (45%), ALT (45%) and AST (34%) in sera of rat group received kojic acid (C1/T) as compared to control rat group (C1). The rat received kojic acid (100mg/kg b.w.) showed higher decrease in the levels of ALP (42%), ALT(44%), and AST(35%) in

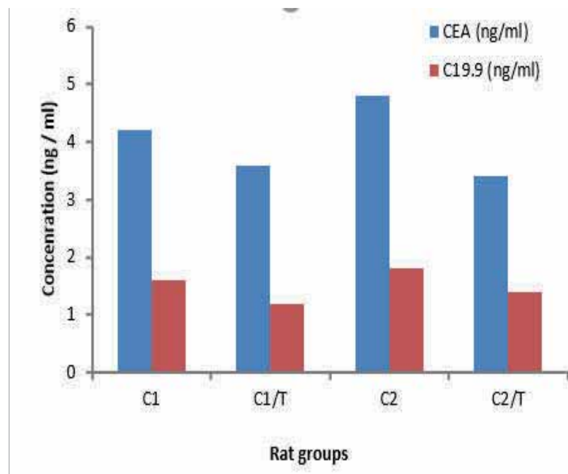

FIGURE 3
CEA and C19.9 levels in sera of all rat groups.

sera of rat group (C2/T) as compared to control rat group (C2). However, AST level was nearly similar decreased in sera of both treated rat groups given kojic acid (C1/T and C2/T).A marked reduction was observed in the levels of γ-GT levels (48 and 54%), in sera of rat groups treated with kojic acid (C1/T and C2/T) compared to their control rat groups (C1 and C2). Highest significant decrease (54%) in the levels of γ-GT was observed in sera of rat groups (C2/T) administered kojic acid (Table 1) more than that of C2/T as well as compared to control rat groups. Similar results were reported by other investigators used different materials [10, 27, 31,41]. The results of the present study indicated that, the kojic acid dose (200mg/kg b.w.) leads to improve the ALP, ALT and AST levels in rat groups received kojic acid (C1/T and C2/T). These results are consistent to other studies [10,39,41]. Moreover, the values of ALP, ALT and AST of rats administered DMH or DEN reflected their abnormal liver function. On contrast the value of ALP, ALT and AST activities in the sera of rats given kojic acid (C1/T and C2/T) reflected their improvements of liver function. Raju et al. [50], observed a significant inhibition of the initiation and development of colon cancer considering that a large portion of the population at risk for colon cancer is characterized by the presence of polyps and large aberrant crypt foci (ACF) in their colons. Higher significant increases were observed in the levels of total protein (Table 1) in sera of rats groups administered kojic acid (C1/T and C2/T) compared to control rat groups (C1 and C2). Significant increase was observed in the level of total protein in sera of rats administered kojic acid (C2/T) more than those of rats administered kojic acid (C1/T). Insignificant differences in albumin and globulin levels were observed. The present results are in accordance with those reported by other investigators [5,10,43]. The present results (Figure 3) showed that the levels of CEA and CA/19.9 were decreased in the sera of rats administered kojic acid (C1/T and C2/T) compared to control rat groups (C1

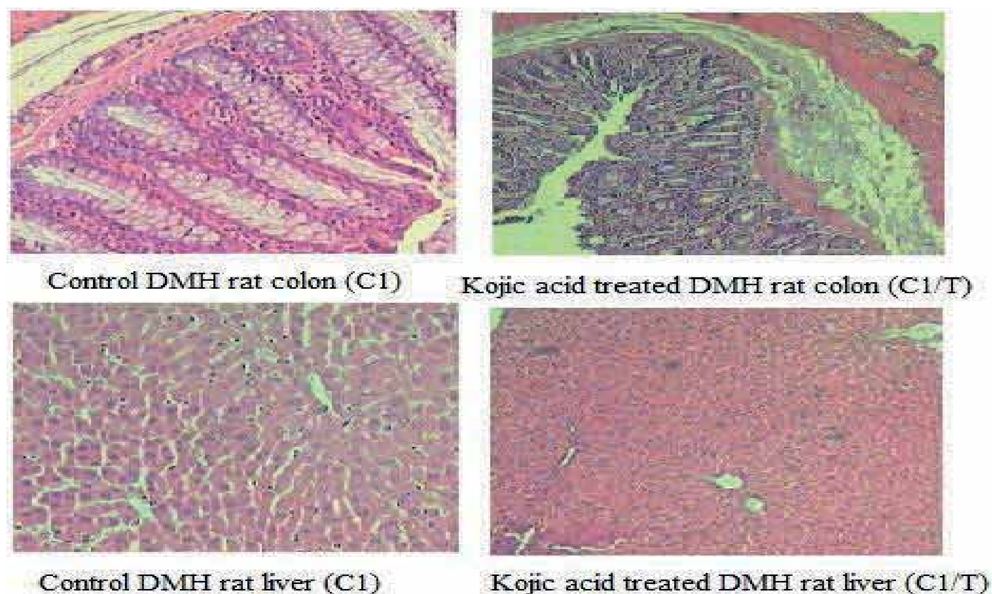

FIGURE 4
Sections of colon and liver from DMH induced rat group (C2) and treated rat group with kojic acid (C2/T).

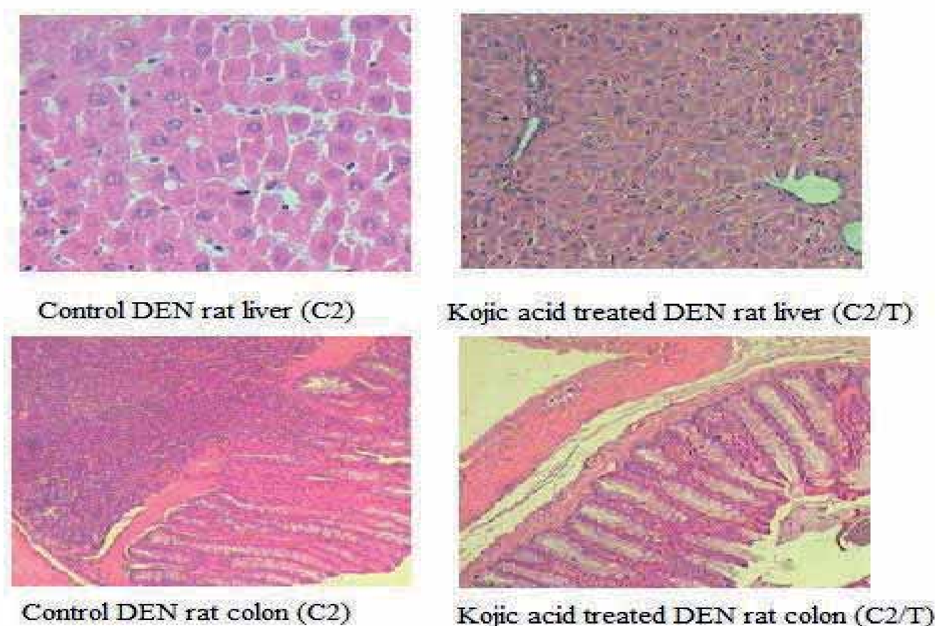

FIGURE 5
Sections of colon and liver from DEN induced rat group (C2) and treated rat group with kojic acid (C2/T).

and C2). Higher decrease was observed in the levels of CEA (Figure 3) in rats administered kojic acid (C2/T) more than those of rats administered kojic acid (C1/T). Insignificant difference was observed in the levels of CA/19.9 (Figure 3).These results are in the range with those reported by other investigators [10,43].

Histopathology. Examined sections of DMH rat colon carcinogenic revealed necrosis of the intestinal and lymphocytes associated with fibrosis [43,45]. DMH control rat colon (C1) illustrated that the lamina propria showed inflammatory cells, lymphoid follicle hyperplasia with degenerative changes (Figure 4). Colon from C1/T group showed normal mucosal lining the submucosa, musclosa and serosa are within normal with no pathological changes (figure 4). Control DMH rat liver tissue (C1) showing mild focal sinusoidal dilatation and mild folicle while kojic acid rat liver tissue (C1/T), showing normal lobular hepatic architecture (Figure 4).Similar results were reported by other investigators [10,31,43] Results showedthat the treatment rat with kojic acid (C1/T) causes some improvement in both histological changes (Figure 4).

Section from liver tissuesof rat received DEN (C2)showing disturbed lobular hepatic architecture, inflammation and hepatocytic with both degenerative and dysplastic nuclear changes (Figure5).DEN treatment usually gives rise tohistological appearances of fibrosis, liverhepatocellular adenoma and hepatocellular carcinoma [26,30,69]. Section from rat colon (C2) showing lymphoid follicle hyperplasia with infiltration of the covering mucosa (Figure 5). The kojic acid rat colon (C2/T) section showing normal mucosal lining and no pathological changes was observed (Figure 5).Similar results were reported by other investigators at the end of 14-26 weeks [5,32,42]. In the present study, rat liver in the groupof kojic acid treatment(C2/T)exhibited reduced proliferation and delayed progression of liver fibrosis at the end of20 weeks compared to control (C2).

The histopathological studies indicated that the kojic acid improved the histology in treatment rat groups(C1/T and C2/T) at doses (40mg/kg) after 20 weeks compared to control rat groups received both carcinogen DMH and DEN (C1 and C2).Based on the present results, it can be concluded that Kojic acid can be considered to have anticancer potential in vitro and vivo. The present study establishes that kojic acid has appreciable anti-cancer activity on different carcinoma cells in vitro and in vivo using rat with chemically induced colon and liver cancers. However, administration of kojic acid to man is simple, since, they are used as common dietary constituents in many parts of the world.

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OIL YIELD AND FATTY ACIDS PROFILE OF COCKLEBUR (*XANTHIUM STRUMARIUM*) AS AFFECTED BY APPLYING DIFFERENT NITROGEN FERTILIZERS

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ABSTRACT

Cocklebur (*Xanthium strumarium*) seeds could be considered as a non-conventional source of oils and fats because of its high lipids content. The aim of this work was to study the effect of using different nitrogen (N) fertilizers [urea (U), ammonium sulphate (AS), and ammonium nitrate (AN)] on the oil content and fatty acid composition of *X. strumarium* seed oil. Nitrogen fertilization relatively affect oil content and fatty acid composition. Samples treated with U20 (urea as 200 kg ha^{-1}) had the highest oil content. The oil contents of other samples showed similar values. *X. strumarium* seeds contained *ca*. 90% unsaturated fatty acids. Linoleic acid was the most abundant fatty acid (*ca*. 70% of total fatty acids), followed by oleic acid (*ca*. 19%). Apart from AN20 and AS10 samples, the other samples showed similarity in the fatty acid profiel wherein linoleic and oleic acids were the main fatty acids. AN20 and AS10 samples had higher values of oleic acid, while linoleic acid exhibited lower content in these samples.

KEYWORDS:
Xanthium, Asteraceae, Compositae, urea, ammonium sulphate, and ammonium nitrate, fatty acid, cultivation

INTRODUCTION

The genus Xanthium (Asteraceae, Compositae) exhibits a global distribution. Xanthium species are used as a herbal drugs for a long time in oriental countries. Cocklebur (*Xanthium strumarium*) grows in the southern and middle bands of Russia, Siberia, and Central Asia. *X. strumarium* inhabits moist sandy soil along banks of rivers and ditches [1, 2]. *X. strumarium* is an annual 30-120 cm in height and is a short-day plant that flowers in July-August. Cocklebur is considered a wild plant and undesirable weed that causes crop yield reduction [3, 4]. Each cocklebur bur contains two seeds. The seeds are covered by a hard-green husk with hooked spines [1].

X. strumarium is recognized as a possible valuable source of medicinal formulations containing iodine, oil, and phenolics [1, 5]. The leaves, stems, fruit, and roots are the used parts of *X. strumarium*. The whole plant has been used to treat diabetes, bacterial infections, and inflammatory diseases [6]. *X. strumarium* is included in traditional Chinese medicine for anticancer treatment [7]. *X. strumarium* whole plant extracts have shown *in vitro* antimitotic trait. Also, *X. strumarium* extract inhibits mammalian cell proliferation through mitotic spindle disruption mediated by xanthatin [2]. A xanthanolide sesquiterpene lactone isolated from *X. strumarium*, has antitumor activity [8].

Turkey has a big agricultural potential with its 25 million hectares of arable land [9]. The above-ground part of *X. strumarium* is considered as a waste material, and this raw material has not been used as a potential renewable source for oil production. Recently, Klimakhin et al. [1] reported that *X. strumarium* seeds contain up to 40%, wherein polyunsaturated fatty acids (PUFA) were the most abundant among fatty acids. However, no other reports were published on the effect of fertilization on the oil yield and fatty acid profile of *X. strumarium* seed.

Nitrogen (N) fertilizers were used for a long time to improve crops yield. Common used N fertilizers are ammonium nitrate (AN), ammonium sulfate (AS), and urea (U). The suitable N dose and source are vital to obtain favourable crop yield and also to obtain high quality oil [10]. Nitrogen influences the fatty acid composition of some oilseeds. For example, N affects the levels of unsaturated fatty acids (i.e., oleic and linoleic acids) in sunflower and linseed oils [11, 12]. Nitrogen dosage also influences the oil yield. Oil yield of rapeseed increased when the N dosage increase [13]. In rapeseed, when different N fertilizers applied, AS and U gave higher oil yield than AN [14]. The fatty acid composition of rapeseed oil

changed when different N fertilizer dosage was used. In rapeseed, low dosage (84 kg N/ha) usage increased total unsaturated fatty acid content and decreased the oleic/linoleic and linolenic ratio than high N dosage [15].

The goal of this investigation was to study the effect of using different N fertilizers (U, AS, and AN) on the oil yield and fatty acid profile of *X. strumarium* seed oil.

MATERIALS AND METHODS

Plant material and growing conditions. Field experiment, employing a randomized block design, were conducted to investigate the interactive effect of 3 different N fertilizers (U, AS, and AN) with two doses (100, and 200 kg ha^{-1}) on the oil and the fatty acid profiles of *X. strumarium* seed oil. Cocklebur seed were collected from Muslubelen gateway (altitude 1440 m) in Yozgat province (Turkey) in 2014. The plants were grown on a sandy loam soil at the experimental field of Bozok University, Yozgat, Turkey (39.753722 N, 34.802738 E, altitude 1267), during spring season. Three replications were made of each treatment. The plot size was 15 m^2 (3 m × 5 m) with 5 rows, with a row-to-row distance of 60 cm. Phosphorous and potassium were applied at the rate of 60 kg ha^{-1} each to all the plots as a basal dressing, while N was given in split doses. The sowing, emergencing and harvesting dates were recorded as on 31 March, 20 May, and 29 September, respectively. Nitrogen fertilizers were given at a time in plants with 3-4 leaves (16 June), and basal dressing was given before sowing. After N fertilization, all experiment area was irrigated once. After plant maturated, they were harvested by hand.

Oil content and fatty acid composition. The seeds of cocklebur were finely ground and the total lipids of seeds was extracted using *n*-hexane in a Soxhlet apparatus for 4 h. After the solvent was removed using rotary evaporator, the extract was dried under nitrogen. The extracted oils were kept in brown bottles, flushed with nitrogen and stored at -18°C until analyses.

Fatty acid methyl esters (FAME) were prepared according to the AOAC [16]. The FAME were identified by Shimadzu (Kyoto, Japan) gas chromatography equipped with Rtx-2330 capillary column (60 m x 0.25 mm i.d., 0.20 µm film thickness) and FID (flame ionization detector). The temperature for the injector was 250°C and the temperature for the detector was 260°C. The oven temperature was held at 140°C for 5 min, then increased to 240°C at 4°C/min and held at 240°C for 20 min. Helium at a flow rate of 1.0 mL/min was used as a carrier gas. A sample of 1 µL was injected by the autosampler with a split mode (split ratio of 1:100). The FAME were identified by comparison with standards and were quantified by the area percentage of each FAME. FAME standards were purchased from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany).

Statistical analysis. Analyses of variance (ANOVA) were performed using SPSS packaged program to determine N fertilizer treatment effects on oil quality and fatty acid composition of seeds. All statistical comparisons were made at α = 0.05 probability level.

TABLE 1
Oil content of *X. strumarium* seeds from plant treated with different rates of N fertilization

	Oil content (%)*
Control	31.48 ±1.36b
U10	29.89 ±1.21b
U20	35.24 ±1.45a
AS10	31.30 ±0.78b
AS20	29.46 ±1.21b
AN10	30.98 ±1.05b
AN20	31.04 ±1.30b

*mean of three determinations ± Standart deviation
Values within the same column not followed by the same letter differ significantly ($p < 0.05$).

RESULTS AND DISCUSSION

The oil contents of cocklebur seeds are shown in Table 1. Oil content of seeds reanged between 29.4% (AS20) and 35.2% (U20). High levles of U fertilizer increased the oil content of *X. strumarium* seeds. According to statistical data, only one sample (U20) was different than others. Except of U20, oil content was not affected by the different N treatments. The resutls are in line with Cheema et al. [17] who found no differences in the oil content of canola seeds treated with different N fertilizers. In addition, our resutls agreed with Dordas and Sioulas [18] who reported that the oil content of safflower seeds were increased slightly when N fertilizer increased but differences between samples were statisticaly insignificant.

The fatty acid composition of *X. strumarium* seed oils as affected by different N fertilization is shown in Table 2. Thirteen fatty acids of cocklebur seed oils were identified, 7 of them were saturated fatty acids (SFAs), 4 were monounsaturated fatty acids (MUFAs), and 2 were polyunsaturated fatty acids (PUFAs). *X. strumarium* oil contained high level of unsaturated fatty acids especially PUFAs. The total content of PUFAs changed between 69.1-73.6%. The predominant PUFAs in *X. strumarium* oil was linoleic acid (C18:2) with levels accounted for 68.3% and 73.5% in AN20 and AN10 treated samples, respectively. MUFAs accounted for 16.8% and 20.8% of the total amount of identified fatty acids. Oleic acid (C18:1) was the main fatty acid among MUFAs and its percentage changed from

TABLE 2
Fatty acid composition of *X. strumarium* seeds from plant treated with different rates of N fertilization*

	Control	U10	U20	AS10	AS20	AN10	AN20
Saturated fatty acids							
C14:0	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00
C16:0	6.03±0.25bc	5.96±0.06c	5.96±0.06c	6.32±0.04ab	6.01±0.11c	5.81±0.15c	6.37±0.08a
C17:0	0.05±0.01bc	0.04±0.00c	0.04±0.00c	0.05±0.00ab	0.04±0.00c	0.04±0.00c	0.06±0.01a
C18:0	2.58±0.18	2.71±0.04	2.54±0.11	2.61±0.41	2.40±0.11	2.71±0.27	2.69±0.01
C20:0	0.13±0.01	0.13±0.01	0.12±0.00	0.13±0.01	0.12±0.01	0.13±0.01	0.13±0.01
C22:0	0.64±0.03abc	0.68±0.00a	0.65±0.02abc	0.60±0.01bc	0.65±0.04ab	0.66±0.04ab	0.58±0.00c
C24:0	0.14±0.00ab	0.15±0.00a	0.15±0.01ab	0.14±0.01ab	0.15±0.01ab	0.14±0.01ab	0.13±0.00b
Total SFAs	**9.58**	**9.70**	**9.48**	**9.87**	**9.38**	**9.51**	**9.98**
Monounsaturated fatty acids							
C16:1	0.08±0.00a	0.08±0.00a	0.08±0.01ab	0.07±0.00ab	0.08±0.00a	0.08±0.01ab	0.07±0.01b
C17:1	0.03±0.00	0.03±0.00	0.03±0.00	0.04±0.01	0.04±0.01	0.03±0.00	0.04±0.01
C18:1	17.98±2.28bc	16.60±0.04c	17.57±0.09c	20.23±0.84ab	17.52±0.18c	16.59±0.68c	20.63±1.18a
C20:1	0.13±0.00ab	0.12±0.00b	0.13±0.00ab	0.13±0.01ab	0.13±0.00ab	0.12±0.01b	0.15±0.01a
Total MUFAs	**18.22**	**16.83**	**17.80**	**20.46**	**17.76**	**16.82**	**20.87**
Polyunsaturated fatty acids							
C18:2	71.90±2.57a	73.33±0.05a	72.56±0.21a	69.00±0.49b	72.68±0.16a	73.53±0.48a	68.39±1.53b
C18:3	0.32±0.24b	0.16±0.03b	0.19±0.05b	0.68±0.02a	0.20±0.02b	0.14±0.03b	0.77±0.27a
Total PUFAs	**72.22**	**73.49**	**72.74**	**69.68**	**72.87**	**73.67**	**69.16**

SFAs−saturated fatty acids; MUFAs−monounsaturated fatty acids; PUFAs –polyunsaturated fatty acids.
*mean of three determinations ±Standart deviation
Values within the same row not followed by the same letter differ significantly ($p < 0.05$).

16.5% to 20.6% in AN10 and AN20 treated samples, respectively. SFAs content of *X. strumarium* seed oils were present in lesser amount and ranged between 9.38 and 9.98 % of total fatty acids. Palmitic acid (C16:0) was found in higher concentration among SFAs, representing 5.81%-6.37% of total fatty acids.

The level of AN influenced greatly the levels of linoleic and oleic acids. Compared with control sample, the increase in AN level resulted in an increase in the oleic acid content but linoleic acid content decreased. In addition, the level of oleic acid increased while linoleic acid level decreased with AS concentration decreased. The similar results were reported for fatty acid composition of rapeseed treated with N fertilizers. Gao et al. [15] reported that a decrease was observed in oleic acid level and an increase in the linoleic acid level compared with control sample for rapeseed treated with N fertilizers. The results of oleic and linoleic acids are consistent with previous report for *Nigella sativa* seed oil treated with varying levels of N fertilizers [19].

CONCLUSIONS

The present study demonstrated that *X. strumarium* seeds are rich in oil. Only urea application with 20 kg/da increased the oil content of cocklebur seeds. Fatty acids of *X. strumarium* oil are comprised mostly of linoleic acid, followed by oleic acid. Seed oil is valuable for unsaturated fatty acids that contribute to human health. Besides, N fertilizition affected the oil content and the quality of the seeds. AN20 and AS10 treated samples showed high values of oleic acid. On the contrary, these samples displayed lower values for linoleic acid. The results showed that cocklebur oil had an economical value and could be used in different industrial appliaction such as pharmaceutical, biodiesel, paint and coatings.

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EFFECT OF PROCESSING METHODS ON MICROBIOLOGICAL AND NUTRITIONAL QUALITIES OF TIGERNUT - FORTIFIED WEANING FOOD.

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ABSTRACT

Traditional weaning foods are known to be of low nutritive value. Thus this work assesses the ability of traditional processing (steeping, malting, roasting, milling) and fermentation methods in enhancement of nutritional qualities of cereal-tigernut blends. Maize fortified with tigernut in ratio 70:30 was processed to produce complementary foods. The formulated blends were allowed to ferment spontaneously for 72 h and effect of different processing methods on microbial load, nutritional quality and the physicochemical parameters were analyzed. The highest lactic acid bacteria count ($5.53x10^{14}$cfu/ml) on MRS agar was recorded in sample C (dry-milled steeped, malted maize and dried roasted tigernut) at 24 h and the least count in the same sample at 0 h. The highest aerobic bacteria count ($2.0x10^{12}$ cfu/ml) was recorded in unfermented sample A (wet-milled steeped maize and tigernut) and the least count ($1x10^{10}$cfu/ml) in unfermented sample B, (wet-milled steeped maize and roasted tigernut) and 72 h fermented sample A and C. Highest yeast count in all sample was recorded at 48 h and mould by 24 h fermentation time. The pH of all the fermented blends decreased drastically within the first 24 h and gradually till the end of time 72 h while the percentage titratable acidity increased with increase in fermentation time, with sample B having the highest TTA (2.2mg/ml). Analysis of proximate content shows that highest crude protein (4.26%) and ash (0.98%) contents and the least contents of fat, fibre and carbohydrate was recorded in sample C while the least protein (3.12%) and ash (0.78%) contents was observed for sample B. Mineral contents were highest in sample B, followed by sample A while sample C had the least contents of all the minerals analyzed. The highest antinutrient contents was observed in sample A while the least was recorded with sample B except for phytate content which was least in sample C. The highest viscosity (1.39ml/mm) and bulk density (1.0408g/ml) contents was observed in sample A and the least in sample C (0.99ml/mm, 1.024g/ml).

KEYWORDS:
Cereal-tigernut, Microbial load, Milling, Roasting, Fermentation.

INTRODUCTION

The vulnerability of infants to problems associated with weaning process is globally concerned, but more importantly in economically developing countries [1]. According to Cameron and Hofvander [2], the weaning period is a crucial event in an infant's life due to the inability of the mother's milk to adequately meet nutrient needs, thus the critical period for developing childhood malnutrition coincides with the introduction of complementary foods which are nutritionally inadequate in many developing countries [3].

The fortification of plant-based complementary foods can be an effective strategy for addressing childhood malnutrition in developing countries [4] provided that it is affordable for most of the population. Traditional weaning foods in West Africa are known to be of low nutritive value [5], and are characterized by low protein, low energy density, and high bulk. Maize pap or koko has been implicated in the aetiology of protein-energy malnutrition in children during the weaning period [6, 7], thus to alleviate the problem of food supply and malnutrition, the development and use of nutritious foods, conventional and non conventional has been advocated [8].

Processing technique such as soaking, roasting and germination are means of improving the nutritional value and protein digestibility of food [9, 10]. Soaking under optimal conditions activates naturally occurring phytases in cereals and results in varying degrees of phytate hydrolysis depending on the kind of cereals [11], also some polyphenols and oxalates that inhibit iron and calcium absorption, respectively, may also be lost by soaking [12], while roasting improve the flavor and palatability of the food product and increase its nutritional bioavailability by inactivating antinutritional factors [13, 14]. According to Gernah *et al.* [15]

Different Treatment And Processing Methods Of The Samples

Sample Code	Treatment	Processing
A	Steeped maize + Steeped tigernut	Wet milling
B	Steeped maize + Roasted tigernut	Wet milling
C	Steeped, malted and dried maize + Roasted tigernut	Dry milling

germination and fermentation of white maize results in the enhancement of its nutritional quality, significant increase in quantity of protein, reduced bulk/viscosity and increased nutrient density as well as reduction in toxins and anti-nutritional factors. Soaking, germination and fermentation leads to a reduction in phytic acid and increases the minerals solubility in foods and could thus improve bioavailability of minerals in cereals and legumes [16].

Tigernut (*Cyperus esculentus*) is a lesser known readily available crop. It is known in Nigeria as ''Aya'' in Hausa, "Ofio'' in Yoruba and ''Akihausa'' in Ibo. The suitabilty of tigernut in formulation of weaning food has also been reported [17]. The high energy, resulting from the rich protein, fat and sugar content, as well as minerals (phosphorus, potassium), vitamins C and E constituents, soluble glucose and oleic acid makes it ideal for infants. Also, tigernut is gluten and cholesterol free and has very low sodium content [18]. It has enormous health benefit because of its high dietary fibre content, which could be effective in the treatment and prevention of many diseases including colon cancer, coronary heart diseases, obesity, diabetics and gastro intestinal disorders [19].

MATERIALS AND METHODS

Collection of Sample. The grain of maize (*Zea mays*) and tigernut (*Cyperus esculentus*) were obtained from Apata Market in Ibadan, Oyo State, Southwestern Nigeria.

Sample Treatment and Formulations. The maize and tigernuts were sorted manually to remove stones, dirt, broken maize, infested nuts and other foreign particles. The cleaned samples were subjected to different processing methods and treatments. The treated samples were formulated in the ratio 70:30 [20] and processed as shown below. Each treated and processed blend was sieved and fermented.

Fermentation. Each formulated blend Sample (A, B and C) was subjected to spontaneous fermentation at room temperature for 72 h except blend C which was reconstituted with distilled water at a concentration of 30% (w/v) [21] before fermentation. At regular intervals during fermentation (0, 24, 48 and 72 hours), samples were taken from the fermenting blends to assess the microbiological, nutritional and physicochemical qualities of the fermenting slurry.

Chemical analysis. Determination of pH. Ten milliliters of steep water and the fermenting gruel were aseptically removed and mixed with 90millilitres of distilled water in a conical flask and its pH determined using the glass electrode of the pH meter (Jenway 3520).

Determination of Total Titrable Acidity. The production of lactic acid was determined by pipetting 10ml of supernatant from the above mixture of steep water and fermenting gruel and titrated against 1N NaOH to phenolphthalein end point. The titratable acidity was calculated as percentage lactic acid (v/v) as each milliliter of 1N NaOH is equivalent to 90.08mg of lactic acid [22]. A colour change from colourless to pink indicated the end point.

Proximate Analysis of Fermenting Blends. Proximate composition (crude protein, crude fat, crude fiber, fat, ash carbohydrate and moisture content) of all the fermenting samples were analysed according to the official methods of analysis described by the Association of Official Analytical Chemist [23]. The ash was determined by incineration of 2g of samples at 550°C until ash was obtained. Protein (N x 6.25) was determined by the macro-Kjeldahl method. The fat composition was determined by exhaustively extracting 2g of sample with petroleum ether. Moisture content was determined by drying the sample in the oven and cooled in desiccator overnight at room temperature and dry matter was then calculated. Total carbohydrate was calculated by difference.

Determination of Mineral Content of Fermenting Blend. Mineral contents of each fermenting blend were determined using Atomic Absorption Spectrophotometer according to the method of AOAC [22]. The minerals were analyzed using the solutions obtained by dry-ashing of 5 ml of the sample at 550 °C and dissolving it in 2 ml of HNO3 and diluted HCl, boiling, filtering and making up to standard volumes with distilled water. The elements (Zn, Fe, Mg, and Ca) were determined by AAS analysis (Philips PU-9100X). The potassium (K) and sodium (Na) were determined by flame photometry using KCl to prepare a standard while phosphorus (P) was determined calorimetrically

using a Spectronic 20 (Gallenkamp, London, UK) instrument with KH_2PO_4 as a standard. All determinations were made in triplicates

Determination of Anti-nutritional Factors. Phytic acid content was determined by employing the procedures of Mega [24]. Tannin and polyphenol contents were determined by the method of Swain [25].

Sensory Evaluation of the Fermented Blends. Sensory characteristics of the developed fermented weaning foods were assessed by 10 untrained panelists of nursing mothers from Ido local government area, Ibadan, Oyo state. The samples were assessed for color, taste, flavor, aroma, texture and over all acceptability. The judges were instructed to sip water before and after assessing each product. The samples were assessed using a 9 point hedonic scale ranging between 9 (like extremely) to 1 (dislike extremely).

Data Analysis. All the data obtained were analyzed using descriptive statistic and the mean scores differentiated using one way analysis of variance (ANOVA). The mean and test significance were determined at $p \geq 0.05$.

RESULTS

The highest lactic acid bacteria count was recorded in Sample C (5.53×10^{14}CFU/ml) at 24 h and the least count in the same sample at 0 h. Colony count on nutrient agar for aerobic bacteria showed that sample A had the highest count of 2.0×10^{12}CFU/ml at 0hr and the least count (1.0×10^{10}CFU/ml) in sample B and C at 0 h, while the highest yeast count was recorded in Sample C (2.15×10^{12}CFU/ml) at 48hrs and the least (1.0×10^{10}CFU/ml) recorded in sample A at 72 h.

Chemical analysis of fermenting blends. Table.1 shows that the pH of all the fermented blends decreased drastically within the first 24 h and gradually till the end of fermentation time (72 h). The least pH of all the sample was observed at 48 h with sample B having the least pH (3.2).The titratable acidity increases with increase in fermentation time with sample B having the highest lactic acid content at most times. The TTA value ranges from 0.3mg/ml to 2.2mg/ml with sample B having the highest lactic acid by 72 h.

Proximate Composition of the Fermented Blends. Table 2 shows the result of the effect of processing methods on the proximate composition of the fermented blends. Sample C had the highest protein (4.26%) and ash (0.98%) contents and the least content of fat (0.28%), fiber (0.26%) and carbohydrate (94.19%),while the least protein content was recorded in sample B (3.12%) which was not significantly different from that of sample A (3.38%). Statistical analysis revealed that the crude protein, ash and moisture content of sample A and B were not significantly different ($p \geq 0.05$) from each other while processing methods significantly ($p \leq 0.05$) affect their crude fat and fiber contents.

Effect of Processing Methods On The Mineral Contents Of The Fermented Blends. Table 3 shows the results of the analysis of the effect of processing methods on the mineral contents of the formulated fermented blends. From the table, the highest mineral content was recorded in wet milled, steep maize and roasted tigernut (Sample B) while the least mineral content was observed in dry milled, steeped, malted maize and roasted tigernut (Sample C). Statistically, processing methods significantly ($p \geq 0.05$) affect the mineral contents of the blends except for the calcium contents of sample A (0.031mg/kg) and C(0.027mg/kg), which were not different statistically ($p<0.05$) but were significantly different from the calcium content of sample B(0.047mg/kg).

TABLE 1
Effect of Fermentation Time on pH and Total Titratable Acidity of the Fermented Blends

Sample code	Parameter	Fermentation time (hrs) 0	24	48	72
A		5.8	3.4	3.3	3.5
B	pH	6.0	3.4	3.2	3.3
C		5.8	3.6	3.4	3.5
A		0.3	0.8	1.3	1.7
B	TTA(mg/ml)	0.3	0.9	1.2	2.2
C		0.2	0.8	1.1	1.5

KEY: Sample A: Steeped maize + steeped tigernut + wet mill
Sample B: Steeped maize + Roasted tigernut + wet mill
Sample C: Steeped, malted, dried maize+ roasted tigernut + dry mill

TABLE 2
Effect Of Processing Method On Proximate Content Of The Fermented Blends.

Sample Code	Proximate Content (%)					
	Crude Protein	Crude Fat	Crude Fiber	Ash	CHO	Moisture
A	3.38±0.09^{b}	0.44±0.02^{a}	0.44±0.03^{a}	0.83±0.02^{b}	94.91±0.10^{b}	84.17±0.82^{a}
B	3.12±0.11^{b}	0.36±0.02^{b}	0.34±0.03^{b}	0.78±0.02^{b}	95.40±0.13^{c}	82.53±0.67^{ab}
C	4.26±0.09^{a}	0.28±0.02^{c}	0.26±0.02^{c}	0.98±0.02^{a}	94.19±0.11^{c}	80.67±0.67^{b}

Values in the same column followed by the same letter are not significantly different (P>0.05) from each other, but differ significantly (P>0.05) with values that do not share a similar letter. CHO:- Carbohydrate content.

TABLE 3
Effect Of Processing Methods On The Mineral Content Of The Fermented Blends.

Sample Code	Mineral Content (mg/kg)						
	Na	K	Ca	Mg	P	Fe	Zn
A	0.026±0.002^{b}	0.097±0.002^{b}	0.031±0.002^{b}	0.062±0.002^{b}	0.032±0.002^{b}	1.125±0.154^{b}	6.525±0.213^{b}
B	0.042±0.002^{a}	0.115±0.002^{a}	0.047±0.002^{a}	0.075±0.002^{a}	0.040±0.002^{a}	1.613±0.197^{a}	7.150±0.198^{a}
C	0.020±0.002^{c}	0.087±0.002^{c}	0.027±0.002^{b}	0.056±0.002^{c}	0.028±0.002^{c}	0.813±0.141^{c}	6.050±0.213^{c}

Values in the same column followed by the same letter are not significantly different (P>0.05) from each other, but differ significantly (P>0.05) with values that do not share a similar letter.

Effect of Processing Methods On The Antinutrient Contents Of The Fermented Blends. Figure 1 shows the result of analysis on the effect of processing methods on the antinutrient content of the formulated fermented blends. Wet milled steeped maize and tigernut (Sample A) blend had the highest antinutrient contents, followed by dry milled malted maize and roasted tigernut (Sample C), while wet milled steeped maize and roasted tigernut (Sample B) had the least content. Samples A and B had the highest Phytate contents (0.0027mg/kg) and the least (0.0023mg/kg) recorded in sample C fermented blend, while polyphenol content were highest in sample A (0.0038mg/kg) and the least in sample B(0.0023mg/kg). The tannin content was highest in sample A (0.0032mg/kg) and the least tannin content in sample B (0.0016mg/kg).

Effect Of Processing Methods On The Viscosity Contents Of The Formulated Fermented Blends. Analysis of the effect of processing methods on viscosity content of the formulated fermented blends as shown in figure 2, that wet milled steeped maize and tigernut (Sample A) had the highest (1.39ml/mm) viscosity contents, followed by wet milled steeped maize and roasted tigernut, Sample B (1.31ml/mm), while the least viscosity (0.99ml/mm) was recorded in dry milled malted maize and roasted tigernut (Sample C).

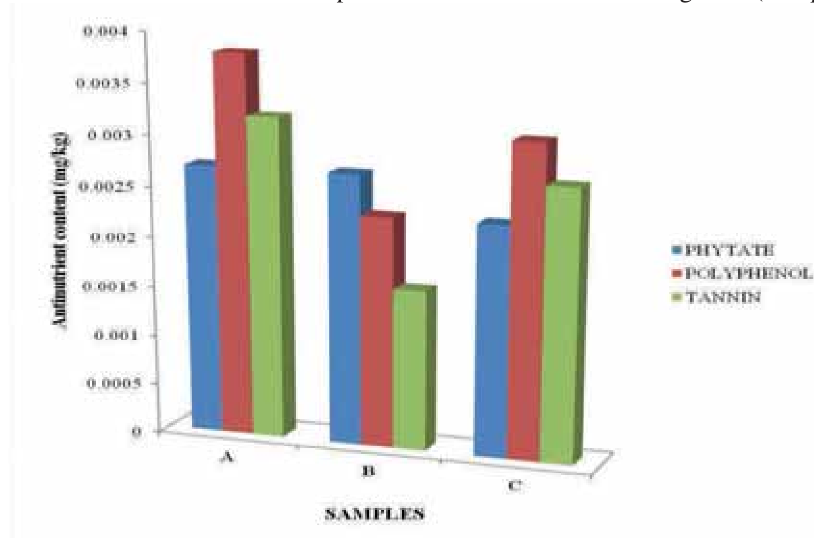

FIGURE 1
Effect Of Processing Methods On The Antinutrient Content Of The Fermented Blends.

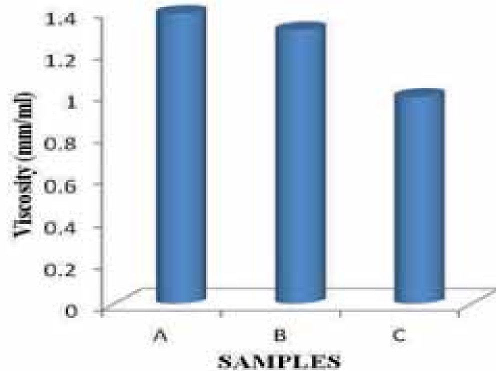

FIGURE 2
Effect Of Processing Methods On The Viscosity Content Of The Fermented Blend

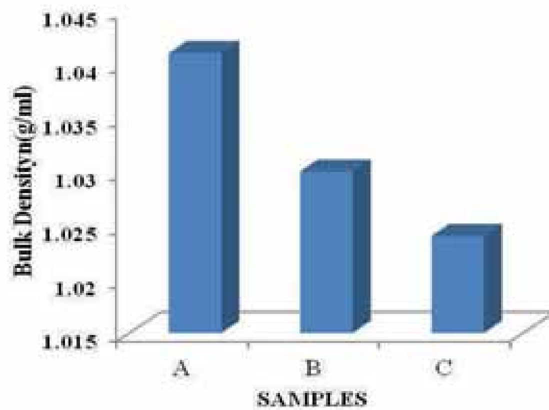

FIGURE 3
Effect Of Processing Methods On The Bulk Density Contents Of The Fermented Blends.

Effect Of Processing Methods On The Bulk Density Content Of the Fermented Blends. Analysis on result of effect of processing methods on bulk density content of the formulated fermented blends as shown in figure 3, revealed highest bulk density content (1.0408g/ml) in wet milled steeped maize and tigernut (Sample A), followed by wet milled steeped maize and roasted tigernut Sample B (1.0301g/ml) and the least in dry milled malted maize and roasted tigernut sample C (1.024g/ml).

Effect Of Processing Methods On Sensory Characteristics Of The Formulated Fermented Blends. Table 4 shows the result of analysis of effect of processing methods on sensory characteristics of the formulated fermented blends. The preference for taste was highest (8.53) in dry milled malted maize and roasted tigernut (Sample C), followed by 7.53 in wet milled steeped maize and roasted tigernut (Sample B), and the least (6.28) in wet milled steeped maize and tigernut (Sample A) with a significant difference ($p \geq 0.05$) among the fermented blends. Evaluation of the colour shows that wet milled steeped maize and tigernut (Sample A) has the highest (8.44) preference rating followed by (7.28) in wet milled steeped maize and roasted tigernut (Sample B), and the least (5.78) in dry milled malted maize and roasted tigernut (Sample C). The colour rating of all the fermented blends were significantly different ($p \geq 0.05$) from each other. The preference for aroma was highest (8.47) in dry milled malted maize and roasted tigernut (Sample C) , followed by 7.72 in wet milled steeped maize and roasted tigernut (Sample B) and the least (6.14) in wet milled steeped maize and tigernut (Sample A). The fermented blends were significantly different ($p \geq 0.05$) from each other with preference to aroma. Evaluation of the texture shows that wet milled steeped maize and roasted tigernut (Sample B) has the highest (8.25) preference rating followed by 7.36 in wet milled steeped maize and tigernut (Sample A), and the least (5.81) in dry milled malted maize and roasted tigernut (Sample C). There was a significant difference ($p \geq 0.05$) among the texture rating of the fermented blends. Acceptability rating shows that dry milled malted maize and roasted tigernut (Sample C) was the most acceptable (8.53), followed by 7.53 in wet milled steeped maize and roasted tigernut (Sample B) and the least acceptability recorded (6.28) in wet milled steeped maize and tigernut (Sample A), with a significant difference ($p \geq 0.05$) among fermented blends.

TABLE 4
Effect Of Processing Methods On Sensory Characteristics Of The Formulated Fermented Blends

Sample	Sensory characteristics				
Code	Taste	Colour	Aroma	Texture	Over-all acceptability
A	6.28 ± 0.61^c	8.44 ± 0.61^a	6.14 ± 0.68^c	7.36 ± 0.64^b	6.28 ± 0.65^c
B	7.53 ± 0.51^b	7.28 ± 0.61^b	7.72 ± 0.57^b	8.25 ± 0.55^a	7.53 ± 0.51^b
C	8.53 ± 0.51^a	5.78 ± 0.59^c	8.47 ± 0.77^a	5.81 ± 0.62^c	8.53 ± 0.51^a

Values in the same column followed by the same letter are not significantly different (P>0.05) from each other, but differ significantly (P>0.05) with values that do not share a similar letter.

Statistical analysis shows that processing methods significantly ($p \geq 0.05$) affect the sensory qualities of the formulated blends.

DISCUSSION

The decrease in pH and increase in titratable acidity as fermentation time increases was possibly because of the accelerated growth rate of lactic acid bacteria which was also reported by Inyang and Idoko [26]. Similarly, Nout *et al*. [27] and Ariahu *et al*. [28] reported that the decrease in pH and increase in titratable acidity may be due to the microbial activity that resulted in the dominance of lactic acid bacteria which degrade carbohydrates resulting in acidification of the product.

Microbiological count shows increase in lactic acid bacteria, aerobic bacteria and yeast count, as fermentation time increases, and a decrease by 72 h in all the samples. Which was similar to the observation by Zvauya *et al*. [29] during fermentation and production of *mangisi*, where the total aerobic mesophilic bacteria count increased with fermentation time. The reduction or total elimination of enteric bacteria and fungi could be as a result of the reduced pH of the medium produced by lactic acid bacteria, which was also reported by Nout *et al*. [30] that lactic acid fermentation exhibits antimicrobial effects on pathogenic microorganisms due to the presence of the acids. Chavan and Kadam [31] reported lactic acid bacteria as one of major microorganisms involved in fermentation of fortified cereal blends. Apart from the flora present on the surface of the grains, microbial flora may have also been established during milling thus explaining the higher initial culturable count in the formulated weaning blends [32].

Proximate analysis of the formulated fermented blends shows a higher value of protein in dried milled steeped malted dried maize and roasted tigernut which was similar to the report of Mba-Anyadioha, [33] who observed that the higher value of protein recorded for fermented maize could be as a result of microbial activities which can cause the *in situ* synthesis of protein and the fact that micro organisms normally use carbohydrates in preference to protein and lipid. The result also agrees with the reports of some authors that the highest protein content observed for sprouted maize might be due to the production of excess amino acids during sprouting and their accumulation in the free amino acid pool used for further protein synthesis 34, 35]. The lower level of crude fat in dry-milled steeped, malted dried maize and roasted tigernut was in agreement with the observation of Inyang and Idoko [26] who also reported reduced fat content in malted millet for "Ogi production. The decrease in fat might be due to the increased activities of the lipolytic enzymes during germination [36], which hydrolyse fats to fatty acids and glycerol while the high ash content was in agreement with Gernah *et al.* [15] who reported that germination of grains leads to increase ash content. Toasting caused an increase in ash content due to volatilization of organic content, thus microbial release of bound minerals will lead to increase in ash content while mineral utilization for the metabolic process of sprouting will cause a reduction in ash content. The lower carbohydrate content of germinated blend might be due to increase in alpha-amylase activity which breaks down complex carbohydrates to simpler and more absorbable sugars which are utilized by the growing seedlings during the early stages of germination.

The observed increase in mineral content in wet-milled steeped maize and roasted tigernut is in agreement with the findings of Osundahunsi and Aworh, [37] who reported that fermentation increased bioavailability of minerals in cereals and legumes via hydrolysis of phytates by microbial phytase leading to the release of divalent cations, such as Ca, Mg, Fe and Zn, bound by phytic acid, while decrease in Na, K, Ca, Mg, P, Fe, Zn, in dry-milled steeped, malted dried maize and roasted tigernut is in contrast to the observation of Kaushik *et al*. [38], who found that germination improves copper (Cu), manganese(Mn), zinc (Zn), riboflavin, niacin and ascorbic acid contents, and the decrease in phosphorus and iron was in contrast to the observation of Saharan *et al.* [39], who recorded that sprouting improve the extractability of Ca, Fe and P to varying extent.

The least phytate content observed in dry-milled steeped, malted dried maize and roasted tigernut was in agreement with the report of Glennie *et al*. [40] that germination of cereal and legumes reduced the level of phytic acid resulting in better nutritional value of sprouts, and also agrees with the work of Onilude *et al*. [41] who recorded a decrease in tannin and polyphenol content due to malting and toasting of cereal/legume blends when compare with sample A (the unmalted and untoasted) while the high polyphenol and tannin contents was in contrast to the observation of Osuntogun *et al*. [42].

The low viscosity in dry- milled steeped, malted, dried maize and roasted tigernut was in agreement to the report of Amankwah *et al.* [43] who recorded that malting alone led to a reduction in product viscosity but fermentation led to an increase. Karlsson and Svanberg [44] also reported reduction in viscosity as a result of enzymes released during fermentation and germination of cereals.

The decrease in bulk density in dry-milled steeped, malted, dried maize and roasted tigernut was in agreement with the findings of Wakil and Onilude [32], who observed that fermentation of malted weaning blends resulted in decrease in bulk density of cereal/legume formulated blends. The high bulk density in samples A and B was in agreement with the findings of Ijarotimi [45] who observed high bulk density in fermented wheat flour.

Assessment of the sensory characteristics showed that dry-milled steeped, malted, dried maize and roasted tigernut (Sample C) had the highest preference in taste, aroma and acceptability, followed by wet-milled steeped maize and roasted tigernut (Sample B) and the least preference in wet-milled steeped maize and steeped tigernut (Sample A) which is in agreement with the observation of Inyang and Zakari, [46] who reported that germinated fura had the highest preference rating, while colour preference was highest in wet-milled steeped maize and steeped tigernut which is also in agreement with the observation of Ocheme and Chinma [47] who reported that the colour of porridge made from soaked millet flour were preferred to those made from germinated millet flour. The texture rating was highest in wet-milled steeped maize and roasted tigernut and least in dry-milled steeped, malted, dried maize and roasted tigernut which is in contrary to the report of Inyang and Zakari [46] who reported that germinated fura had the highest preference rating for texture.

CONCLUSION

The result of the research show that processing techniques such as steeping, roasting, malting, wet and dry milling in addition to fermentation can be used in improving the nutritional, physicochemical and organoleptic properties of formulated fermented weaning blends. Thus fortification of plant-based complementary foods and the use of dry-milled steeped, malted maize and roasted tigernut at a ratio of 70:30 can be used to produce weaning blend of improved nutritional quality which can be an effective food for addressing childhood malnutrition.

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ROLE OF SALINE WATER ON PROLINE, BETAINE AND SOME CHEMICAL MARKERS IN WHEAT (*TRITICUM AESTIVUM*) GENOTYPES

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ABSTRACT

Chemical markers can restrain by accumulation of oxygen free radicals in tissues. This study was conducted to determine the chemical markers |Malondialdehyde (MDA), Di-Hydroxy guanizine (8-oH-dg) and Di-Tyrosine (Di-Ty)| with affected by saline water with split plot arrangement based on randomized complete block design with four replications such as two wheat genotypes (Karaj 2 and Sabalan) was in main plots and ESP [11.5 (E_1), 12 (E_2) and 12.5 (E_3) meq lit^{-1}] was laid on sub plots during 2013 and 2014 cropping year. In this work, content of chemical markers, proline and betaine were investigated in response to saline water in wheat. Karaj 2 had higher levels of proline and betaine and lower levels of markers than sabalan. These results suggested that proline and betaine could regulated the effects of osmotic and oxidative stress related markers that enhance the occurrence of cell injury caused by high salinity, thus betaine improved the salt tolerance in karaj 2. We concluded that markers can stimulate sodium destructive effects in wheat.

KEYWORDS:
ESP, Di-hydroxy guanosine, Di-tyrosine, Malondialdehyde, Salt stress, Wheat

INTRODUCTION

The yield resistance to salt stress can be related to chemical markers. These markers can play an important role to separate of nucleotide strands (DNA and RNA) at high concentration of leaf protein [4]. It generally indicated that a low content of Di-Ty and 8-oH-dg and high grain yield and biomass potential, which are more desirable traits in adaptation to salt stress [17]. Moreover, the great yield potential for wheat adaptation relative to irrigation water when ESP is up to 12 meq lit^{-1} [22], and it is also displays better tolerance to salt stress [16]. Recent evidence however, suggests that the ability of nucleotides is a tool for reduction the effects of saline water in crop plants [24]. Sodium in irrigation water, due to its osmotic properties, has been induced as an alternative to destructive agents in cellular membrane [19] by chemical markers for significant damage; when oxidation has great influence on MDA and proposed to be harmful [23]. In resistant crops betaine and proline content show significantly increased tolerance to salt stress [18]. Thus resistant plants grown in the field, the physiological and biochemical mechanisms by low content of markers and high rates of betaine and proline may improving to salt tolerance, and require further study [14]. There are a few possibilities suggesting that potential of wheat has improved in recent years at high rates of ESP due to decrease activity of 8-oH-dg and tyrosine [9]. Also, innovations have evolved that high rates of ESP have decreased wheat grain yield to the point that the most cereal for feed stocks [27], including in this study, refer to high content of MDA, Di-Ty and 8-oH-dg in leaf protein.

This is important that when ESP of irrigation water was up to 11.8 meq lit^{-1}, the damage of leaf tissue was increased with high rates of Di-Ty and 8-oH-dg [16]. For instance, when Di-Ty was up to 5 nm mg^{-1}, low ability of ribosomes for separation at high rates of Na cause to be damage of DNA and especially RNA and prevent to translate of protein [8]. These results suggested that content of chemical markers, proline and betaine in the plant tissues might be the main factor for resistant crops in saline soils [27].

This work was investigated the effects of chemical markers and betaine in wheat at high rates of ESP in irrigation water. Thus, the objective of this research was to determine the efficiency of chemical markers, proline and betaine in response to saline water. The effect of these techniques was evaluated on grain yield and grain weight in this study.

MATERIALS AND METHODS

This research was conducted in Karaj Research Field during 2013 and 2014 cropping year. The station is located at longitude 46°12' E and latitude 32°41' and 1380 m above the sea level. The soil (Table 1) was plowed in the autumn of previous year by mould board plow and in the spring

TABLE 1
The soil conditions and profile.

Soil depth	Soil texture	pH	EC ($ds.m^{-1}$)	N **(%)**	P $mg.kg^{-1}$	K $mg.kg^{-1}$	CO_3 $mg.kg^{-1}$	HCO_3 $mg.kg^{-1}$
-30	Silty loam	7.42	2.33	0.061	2.98	121	0.22	3.54

TABLE 2
Range of ions in different ESP rate.

	Ca meq lit^{-1}	Mg meq lit^{-1}	CO_3 meq lit^{-1}	HCO_3 meq lit^{-1}
ESP = 11 meq lit^{-1}	5.32	2.44	1.43	9.33
ESP = 12 meq lit^{-1}	13.35	3.78	2.98	16.15
ESP = 12.5 meq lit^{-1}	22.9	7.94	5.11	27.73

two perpendicular discs were used. Before cultivation, 240 kg of phosphorus (based on 120 kg ha^{-1} of triple super phosphate source) and 120 kg of Nitrogen from urea source (250 kg ha^{-1}) were distributed in the land and mixed with soil by disc. Details of the experimented soil are shown in Table 1.

The randomized complete block design using split-plot arrangement with four replicates. Two wheat genotypes [karaj 2 (G_1) and sabalan (G_2)] was arranged in main plot and ESP [11.5 (E_1), 12 (E_2) and 12.5 (E_3) meq lit^{-1}] was laid in sub plot. Plot size was six rows by 0.2 m width and 6.0 m long. The first irrigation obtained with neutral water and was 9 m^3 for each plot. Different rates of ESP were made by additive ions in to water reservoir tanks by [23] method (Table 2).

ESP was calculated by below ratio:

ESP=SARiw (irrigation water) × [1+SI] mili equivalent lit^{-1} $SARiw = \sum_{j=1}^{n} \left[\frac{Na}{\sqrt{Ca + Mg/2}} \right]$ mili equivalent lit^{-1} SAR = Sodium Absorbed Ratio

SI = Saturation Index = 8.4 - pHc

pHc = Corrected pH = (pk_2 _ pk_c) + p (Ca+Mg) + p (Co_3+HCo_3)

(pk_2 _ pk_c) = Ca + Mg + Na mili equivalent lit^{-1} p (Ca + Mg) = Negative logarithm of Ca and Mg concentration

p (Co_3 + HCo_3) = Negative logarithm of Co_3 and HCo_3 concentration

Plants were irrigated by saline water at 3 leave stage. Markers, proline and betaine were assayed at the mid of flowering (85 days after planting). Leaf samples (500 g for each treatment) were homogenized in 1 mL of cold buffer (0.25 M Tris, 0.2 M sucrose, 5 mM dithiothreitol, pH 7.4). After centrifugation, the supernatant was derivatized with thiobarbituric acid and the MDA–thiobarbituric acid complex extracted with butanol. Samples were then analyzed by reverse-phase high-performance liquid chromatography as previously described [1]. Results were adjusted for protein content in the sample and expressed as nanomoles of malondialdehyde and Di-Tyrosine per milligram of protein.

According to the method of [10], leaf samples (500 g for each treatment) were homogenized containing with 0.16 M trisbuffer phosphate (pH 7.5) and then enter disodic phosphate (0.174 mmol lit^{-1}, pH 7.5) and 0.15 M EDTA with carbon column based on LCEC in 25 degree centigrade. Results were adjusted for protein content in the sample and expressed as nanomoles of Di hydroxy guanosine (8-oH-dg) per milligram of protein.

Betaine content was calculated at mid of flowering from Fresh leaves (0.5 g for each treatment) were ground in liquid nitrogen and 25 mL of 95% ethanol was added [15]. After being heated for 3 h, the concentrate was diluted with 1.5 mL HCl and 0.3 mL of petroleum ether was added for extraction. Active carbon was added to decolorize the solution. After centrifugation, the supernatant was heated for 10 min in boiling water. One milliliter of Reinecke's salt was added, and the solution was cooled for 3 h. After centrifugation, the supernatant was precipitated with 1 mL of ethyl ether. The precipitate was re-dissolved in 1 mL of 70% acetone and the absorbance was read at 525 nm and expressed as milligram of gram protein. The betaine content was calculated as follows:

betaine content: $(A_{525}–0.0121)/0.035 \times 1.5 \times 25/0.5$.

Proline contents were determined according to the method of [3]. Wheat leaf samples (0.5 g) from each group were homogenized in 3% (w/v) sulfosalicylic acid, and the residue was removed by centrifugation. The extract (2 mL) was mixed with 2 mL of glacial acetic acid and with 3 mL of acid ninhydrin (1.25 g of ninhydrin was warmed in a mixture of 30 mL of glacial acetic acid and 20 mL of 6 mol L^{-1} phosphoric acid until dissolved) for 1 h at 100 °C; the reaction was terminated in an ice bath. The reaction mixture was extracted with 5 mL of toluene. The chromophore-containing toluene was warmed to room temperature and its optical density was measured at 520 nm and expressed as microgram of gram fresh weight.

Data were analyzed by combine analysis of variance and Duncan's multiple-range tests using SPSS (Statistical Product and Service Solutions) software. Statistical significance was set at $P < 0.05$.

TABLE 3
Combine analysis of variance for the effects of saline water and wheat genotypes on biochemical traits in two cropping year.

S.O.V	df	Proline	MDA	Di-Ty	8-oH-dg	Betaine
		Mean Square				
Year (Y)	1	0.701^{ns}	0.815 ^{ns}	0.635 ^{ns}	0.537 ^{ns}	0.794 ^{ns}
Replication (R)	3	0.578^{ns}	0.994 ^{ns}	0.891 ^{ns}	0.831 ^{ns}	0.870 ^{ns}
Y×R	3	0.547^{ns}	0.648 ^{ns}	0.705 ^{ns}	0.727 ^{ns}	0.644 ^{ns}
Genotype (G)	1	855.14**	1355.17**	674.28**	571.25**	1642.25**
G × Y	1	0.719^{ns}	0.901 ^{ns}	0.803 ^{ns}	0.827 ^{ns}	0.599 ^{ns}
R×G	3	678.91**	1172.44**	501.30**	536.69**	1208.41**
Y×R×G	3	0.624^{ns}	0.802 ^{ns}	0.628 ^{ns}	0.739 ^{ns}	0.845 ^{ns}
ESP (E)	2	605.24**	736.69**	496.71**	441.14**	745.63**
E×Y	2	0.578^{ns}	0.699 ^{ns}	0.515 ^{ns}	0.501 ^{ns}	0.612 ^{ns}
E×R	4	447.96**	651.31**	345.62**	329.55**	0.579**
E×G	2	399.86 **	490.06 **	289.61 **	181.36 **	376.59 **
E×R×G×Y	6	0.379^{ns}	0.858 ^{ns}	0.713 ^{ns}	0.677 ^{ns}	0.711 ^{ns}
CV%		7.69	7.88	6.12	5.44	8.05

** Significant differences at P=0.01 level. ns: Not Significant.

TABLE 4
Mean comparisons of wheat genotypes, ESP treatments and their interaction on biochemical characteristics.

Genotype	ESP meq lit^{-1}	Proline (µg g^{-1} FW)	MDA (nm mg^{-1})	Di-Ty (nm mg^{-1})	8-oH-dg (nm mg^{-1})	Betaine (mg g^{-1})
	2013					
	11.5	50.44 ^{b}	9.77 ^{d}	2.57 ^{d}	1.38 ^{d}	2.55 ^{b}
Karaj 2	12	59.68 ^{ab}	10.65 ^{cd}	3.84 ^{c}	2.40 ^{c}	3.44 ^{ab}
	12.5	70.11 ^{a}	12.36 ^{bc}	4.52 ^{bc}	2.98 ^{bc}	4.61 ^{a}
	11.5	39.61 ^{bc}	11.89 ^{c}	3.37 ^{c}	2.39 ^{c}	1.98b ^{c}
Sabalan	12	44.19 ^{b}	13.57 ^{b}	5.44 ^{b}	3.89 ^{b}	2.84 ^{b}
	12.5	56.97 ^{ab}	16.98 ^{a}	6.15 ^{a}	4.91 ^{a}	3.79 ^{ab}
	2014					
	11.5	52.70 ^{b}	9.91 ^{d}	2.71 ^{d}	1.44 ^{d}	2.63 ^{b}
Karaj 2	12	59.96 ^{ab}	11.05 ^{cd}	3.82 ^{c}	2.45 ^{c}	3.39 ^{ab}
	12.5	70.29 ^{a}	12.53 ^{bc}	4.58 ^{bc}	3.01^{bc}	4.65 ^{a}
	11.5	39.56 ^{bc}	11.95 ^{c}	3.46 ^{c}	2.33 ^{c}	1.94 ^{bc}
Sabalan	12	44.28 ^{b}	13.88 ^{b}	5.38 ^{b}	3.86 ^{b}	2.91 ^{b}
	12.5	57.01 ^{ab}	16.90 ^{a}	6.17 ^{a}	4.97 ^{a}	3.78 ^{ab}

a-d Means followed by the same letter within a column do not differ significantly at P< 0.05 according to Duncan's Multiple Range Test.

RESULTS

Analysis was showed significant differences in all traits but not differ ($p > 0.05$) in either year (Table 3). However, significant difference in interaction effects was due to saline water when genotypes were considered as a main factor in analysis of variance.

As indicated in Table 4, high rates of betaine (4.61 and 4.65 mg g^{-1}) and proline (70.11 and 70.29 µg g^{-1} FW) in karaj 2 at high rate of sodium (E_3) could improve ability of genotypes to salt tolerance throughout the growing season. Because the salt tolerance of karaj 2 was slightly higher than that of sabalan. It was indicated that Di-Ty was more sensitive than 8-oH-dg and MDA in response to sodium. About more content of Di-Ty (23.7 to 26.5%) and 8-oH-dg (42.25 to 39.3%) was found in sabalan than karaj 2 when ESP was 11.5 to 12.5 meq lit^{-1} (Table 4).

Although betaine is critical for osmoprotection, there were significant differences in betaine and markers content between genotypes in response to ESP rates (Table 4). Our results also support prior findings that crop productivity can be affected with ESP rates and duration plant developing stage and these effects were exacerbated to high rates of markers especially MDA (12.53 nm mg^{-1} in karaj 2 and 16.98 nm mg^{-1} in sabalan) under sodium stress conditions (Table 4).

DISCUSSION

Reported that decreased of chemical markers in wheat with greater sodium uptake would support

betaine formation and avoid low potential of yield [2]; [11]. This is indicated that wheat genotype could promote redistribution of assimilates to grain that would augment seed mass and yield formation under sodium stress conditions [13]. Reported that low rates of tyrosine and MDA could be successfully improved stress tolerance and grain quality [5]. This provides evidence that these markers are less harmful if appropriate genotypes are utilized. Ribosomes cannot be separate in response to high rates of tyrosine [21], it was caused to higher betaine and proline content in karaj 2 than sabalan in E3 in two cropping years (Table 4).

Oxidation process in wheat cells is believed to directly impact a range of salts in soil solution [12]. By oxidative reaction, RNA and DNA may change their functions and cause to enhance of respiration [16] and thus their interactions with markers and salt stress can destroy a protein network and it is related to betaine content. Methionine synthase catalyzes the formation of methionine by the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine [6]. This reaction occurs in the activated methyl cycle, which is known as the metabolic source of single carbons. In this cycle, methionine is further converted into S-adenosylmethionine (SAM) by S-adenosylmethionine synthetase [20]. SAM provides a methyl group for many metabolites, including important compounds, such as betaine, methylated polyols, and polyamines, under high salinity conditions. Betaine and methylated polyols are compatible solutes that accumulate in the cytoplasm and that regulate osmotic balance under salt stress [14]; [20]. Thus the up-regulation of methionine synthase in karaj 2 (4.61 to 4.65 mg g^{-1}) may play an important role in improving the ability of wheat to salt tolerate by regulating the osmotic balance (Table 4).

Under salt stress conditions, proline accumulates to high-concentration in cell cytoplasm. However, it has also been suggested that over-accumulation of proline could be toxic to plant cells [25]. There were 39.56 (sabalan) to 70.29 (Karaj 2) µg g^{-1} FW increment in proline accumulation under salt stress conditions (Table 4). It was noticed that average proline content enhanced under salt stress condition in 2 wheat genotypes and cropping years Initial level of proline in non-stressed plants (E_1) was ranging from 50.44 to 52.70 µg g^{-1} FW in karaj 2 and 39.61 to 39.56 µg g^{-1} FW in sabalan, while under stress condition. However, its level has not always been associated with tolerance level. It has been suggested that proline functions as an indicator of plant water status but not a measure of level of tolerance [11] ; [25].

The impact of ESP on markers content and wheat genotype in Table 4 were used to total grain yield based on saline water, with reduction of grain weight. Significant change was found in the total seed weight, but in E_3 had significant changes (Table 3). Overall this indicated a shift towards higher grain weight (karaj 2) and this confirms the previous work by [7] who showed changes in total grain yield proposed to be due to enhance of sodium between markers and betaine subunits [26]. However, both sodium rates of irrigation water and content of markers attribute to stimulate adoption in wheat plants [21]. We observed that high sodium rate could increase chemical markers content (Table 4) at high ESP (12 to 12.5 meq lit^{-1}), suggested that these markers can be remove the nucleotides in plant cells [3].

CONCLUSION

This diagnosis was reduced by using different sodium rates to identify management of salt stress. On the other hand, ESP and genotype had significant difference in this study and sodium rates in irrigation water is important until we can better characterize and control the conditions with associated by chemical markers in wheat under salinity condition (Table 3). This is suggesting that sodium rates in irrigation water are significant sources for chemical markers, proline and betaine. Controlled studies are needed to further validate the interactions which found in our research.

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