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REGULAR ARTICLE

Mango (*Mangifera indica* cv. Azúcar) antiinflammatory and chemopreventive role during colorectal carcinogenesis

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ABSTRACT

Chemopreventive activities of natural compounds result in the modulation of several pathways and molecular targets. It is common to find effective potential candidates for cancer chemoprevention with anti-inflammatory properties. Mango (*Mangifera indica* cv. Azúcar) has shown anticarcinogenic effects and it is a source of bioactive compounds. This study evaluated the effects of mango on Aberrant Crypt Foci formation and inflammatory biomarkers after initiation of colon carcinogenesis in AOM-treated mice. Ripped mango pulp (*Mangifera indica* cv. Azúcar) composition was identified by HPLC. Azoxymethane-treated mice received the fruit (0.3% w/v) for eight weeks and the distal part of colon was collected and stained with methylene blue to look for aberrant crypt foci (ACF); scrapped mucosal cells were processed for prostaglandin E2 detection by ELISA; and blood levels of pro-inflammatory cytokines (interleukin-1Beta, tumor necrosis factor-alpha, interleukin 6) were also assessed by ELISA. Student's *t*.test was used for the comparisons between mango treated and untreated groups. ACF formation was reduced by 67.5% and prostaglandin E2 levels were also reduced in mice which ingested the fruit. Cytokines levels were unchanged by mango consumption. In the chromatography were identified phenolic acids and Beta-carotene. Mango pulp showed chemopreventive effects through the reduction of ACF formation, by means of blocking hyperproliferation which is correlated with decreasing levels of PGE2.

Keywords: Mangiferin indica; Colorectal cancer; Chemoprevention; Prostaglandin E2; Cytokines

INTRODUCTION

Inflammation is a hallmark of carcinogenesis (Hanahan and Weinberg, 2011) implicated in all stages of carcinogenesis, i.e., initiation, promotion and progression (Bousserouel et al., 2010a). An agent that can diminish chronic inflammation has the potential to delay or prevent the onset of chronic diseases as cancer (Aggarwal et al., 2011). The increasing levels of inflammatory factors (cytokines and cellular activation markers) during colon carcinogenesis and risk reduction by non-steroideal anti-inflammatory drugs use have been confirmed by epidemiologic data in humans and experimental data in rodents (Bousserouel et al., 2010a; terzic et al., 2010; Day et al., 2013).

Azoxymethane (AOM) induced colon carcinogenesis in rodents is a useful tool to study the carcinogenic process because it emulates the same changes as in humans, beginning with aberrant crypt foci (ACF) formation considered as a surrogate biomarker for pre-malignant change, providing a simple and economical tool for preliminary screening of potential chemopreventive agents (Tanaka, 2009). AOM treatment also enhances the transcription factor NF-kB (nuclear factor kappa B) levels in colon and expression of the inflammatory enzyme cyclooxygenase-2 (COX-2) (Velmurugan et al., 2010).

Chemopreventive activities of natural compounds result in the modulation of several pathways and molecular targets; as the neoplastic transformation of cells also proceed through a broad kind of cellular, biochemical and molecular changes. Effects on oxidative stress, apoptotic response and inflammation, are important targets for controlling carcinogenesis (Tan et al., 2011). It is common to find

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effective potential candidates for cancer chemoprevention with anti-inflammatory properties as curcumin, resveratrol and lycopene (Armin et al., 2009). *In vivo* models of early modulation of inflammatory markers during colorectal carcinogenesis, have shown changes of pro-inflammatory cytokines interleukin-1beta (IL1β), Tumor Necrosis Factor α (TNFα), enzymes (COX2, inducible nitric oxide synthase (NOS2)) and transcription factors (NFkB) by the consumption of natural chemopreventive compounds (Armin et al, 2009; Bousserouel et al., 2010b; Bousserouel et al., 2011; Kauntz et al., 2012; Velmurugan et al., 2008).

Previous studies indicate that mango is a good source of antioxidant bioactive compounds (Corrales-Bernal et al., 2014; Noratto et al., 2010; Palafox-Carlos et al., 2012), and it prevents initiation of colon carcinogenesis in rodent models (Corrales-Bernal et al., 2014; Boateng et al., 2007). However, there is a lack of information on the chemopreventive effects of this fruit in early post-initiation of colon carcinogenesis and the mechanisms involved. Therefore, we have evaluated the chemopreventive effects of mango after the initiation of colon carcinogenesis in AOM-treated mice that ingested the fruit as a beverage liquid during eight weeks, and ACF formation was assessed. Furthermore, in order to get insight into the mechanisms involved in the chemopreventive effects of mango, several key inflammatory biomarkers (IL1β, TNFα, interleukin 6 (IL6) and prostaglandin E2 (PGE2)) were measured.

MATERIALS AND METHODS

Plant material

Fresh ripe mango fruit (*Mangifera indica,* cv. Azúcar) from the Caribbean coast of Colombia were selected based on the Colombian Standards of Grades (NTC5139) (ICONTEC, 2004). The peel surface color of each fruit selected was 100% yellow and the color parameters obtained were L=22, a=17.3 and b=19. They were then processed to obtain the lyophilized pulp as described in Corrales-Bernal et al. (2014). The pulp was lyophilized at the beginning of the study and it was split in aliquots and stored in dark for all the experiments.

Preparation and composition of mango extracts

Hydrophilic extracts were prepared with 80% methanol/1% formic acid as it was reported by Palafox et al. (2012), and lipophilic extracts were prepared with a mixture of hexane/acetone/toluene/ethanol as reported by Rivera-Pastrana et al. (2010) and Corral-Aguayo et al. (2008). Samples were injected automatically into an HP 1100 series High Performance Liquid Chromatography (HPLC) system (Hewlett–Packard, Palo Alto, USA) equipped with a diode array detector (DAD).

For hydrophlic compounds, the HPLC system was equipped with an Xterra RP18 reverse phase column (4.6 mm; 250 mm; 5μm). The mobile phase was composed of 1% formic acid (A) and acetonitrile (B), and the elution gradient was 2–100% (B) in 60 min. The injection volume was 25 μL. The absorption spectra were recorded at 280 and 320nm.

For lipophilic compounds the HPLC system was equipped with a C30 reverse phase column (4.6 mm; 150 mm; 3 μm) (YMC Inc., Milford, MA), and the mobile phase was composed of methanol (A) and tert-butyl-methyl eter (MTBE) (B), and the elution gradient was 2 to 100% (B) in 55 min. The injection volume was 100 μL, and the absorption spectra were recorded at 450nm.

Chromatographic identifications were based on comparing retention times and on-line ultraviolet and visible absorption spectrum data with authentic standards of gallic acid, coumaric acid, chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, catechin, epicatechin, quercetin, myricetin, pellargonidin, isorhamnetin, kaempferol and *β*-carotene (> 90% purity, Sigma- Aldrich St. Louis, Mo., U.S.A.).

Animals and treatment

All animal experiments were performed in accordance with national and international guidelines (Ley 84/1989, Res. 8430/1993 and European Community Council and Canadian council on Animal Care, 1998) with the authorization of the Ethics Committee for Animal Experiments of the University of Antioquia (No. 68 of 28th June 2011).

Female Balb/c mice at the age of 6 weeks and weighing 16-24 g, were maintained in animal facility at Corporación para Investigaciones Biológicas (Medellín, Colombia), under 22-25°C, 12 h light/12 h dark cycle with free access to food and drinking liquid (water or whole mango juice, ad libitum) during 10 weeks. Twelve mice received intraperitoneal injections of AOM (Sigma-Aldrich, Saint-Quentin Fallavier, France), at a concentration of 15 mg/kg body weight, once a week for 2 weeks. One week after the last injection of AOM (post-initiation), mice were randomly separated into two groups. One group (n=6) received daily a freshly prepared whole mango juice (WMJ) in drinking water (0,3% w/v), while the AOM-control group (n=6) received only drinking water. Mice consumed daily an average of 3,7 mL of mango juice (0,5 g/kg body weight). All animals were sacrificed 8 weeks after AOM injections.

Aberrant crypt foci assessment

ACF analysis was done according to Bousserouel et al. (2012). The most distal part of the colon (4cm in length)

was washed with physiological saline, cut, opened and fixed flat between two pieces of filter paper in 10% buffered formalin for a minimum of 24 h. The colon was stained with 0.2% methylene blue for 5 min, rinsed in Krebs-Ringer buffer, placed onto a glass slide and examined microscopically using a low power objective (10X) to assess the occurrence and multiplicity of ACF. The criteria for the identification of hyperproliferative aberrant crypts were: (i) increased size; (ii) thicker epithelial cell lining; and (iii) increased pericryptal zone relative to normal crypts.

Determination of PGE2 concentration in colonic mucosa

Mucosal scrapings of the distal colon stored at -70°C were used for PGE2 determination. Samples were homogenized in Tris-HCl buffer (100mM, pH: 7,8) containing EDTA (1 mM). After centrifugation at 10000 x g for 15 min at 4°C, the supernatants were collected and PGE2 measured using the PGE2 assay kit (R&D Systems Inc. Lille, France) according to manufacturer's procedure (Bousserouel et al., 2010a).

Determination of inflammatory cytokines

Determination of inflammatory cytokines levels (IL1β, IL6 and TNFα) in serum were evaluated by Multi-Analyte ELISArray kit (Mouse inflammatory cytokines, Qiagen group) according to manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Software (San Diego California, USA). Student's t-tests were used for comparisons between mango treated and untreated groups. For correlation of PGE2 levels with ACF/colon was used the Pearson correlation coefficient

RESULTS AND DISCUSSION

Colorectal cancer chemoprevention is a strategy to reverse, suppress or prevent carcinogenesis at its early stages with the use of pharmacological or dietary interventions (Steward and Brown, 2013; Russo, 2007). Study of chemopreventive potential of dietary factors from fruits has gained interest due to the evidence of reduced CRC risk with consumption of plant-derived food enriched with phytochemicals whose potential to prevent colon cancer is due to the diverse cellular pathways they modulate simultaneously (Boghossian et al., 2012). Mango is one of such fruits, which is a tropical fruit widely accepted by consumers with antioxidant and antiproliferative activities against various cancers (Noratto et al., 2010; Ribeiro et al., 2010).

The aim of the present study was to elucidate the inhibitory role of mango (*Mangifera indica* cv. Azúcar) against AOM induced colonic ACF during the early post-initation stage of carcinogenesis and its association with changes on inflammation related biomarkers in colonic mucosa and circulation to get more insight about chemopreventive potential effect of mango (cv. Azúcar) on colon carcinogenesis.

The regular intake of mango juice (cv.Azúcar, at 0.3 % w/v, 0.5g lyophilized mango/kg body weight) for 8 weeks after injections with AOM, reduced the formation of ACF in the colon by 67.5% ($p = 0.013$) in mice, when compared with the control (AOM, water) receiving only water (Fig. 1a-c). Furthermore, the analysis of crypt multiplicity (number of crypts/focus) indicates that mango also diminished the hyperproliferation of ACF (Fig. 1d). These results, in addition to others obtained in our laboratory (Corrales-Bernal et al., 2014), demonstrate chemopreventive effects of mango at initiation and promotion of AOM-induced colon carcinogenesis in mice with the same mango concentration at 0.3% w/v. Only one report has been found about this same assessment with mango although the cultivar was not mentioned, where it concluded a high inhibition of ACF formation (83.3%) with the consumption of 5% w/w of mango (Boateng et al., 2007).

Inflammation is a hallmark of cancer and is involved in early stages of carcinogenesis (Perwez-Hussain and Harris, 2007). Increasing levels of inflammatory factors such as cytokines and cell activation markers (TNFα, IL1β, proliferating cell nuclear antigen (PCNA), COX-2) has been observed during colon carcinogenesis in rodent models (Tanaka,2009), and some of these inflammatory factors have been proposed as biomarkers in chemoprevention studies (Bousserouel et al.2010a; Bousserouel et al.2010b; Bousserouel et al., 2011; Kauntz et al., 2012). There is still a lack of information about inflammatory cell infiltration which could be useful to increase the understanding of the environmental influences within early pre-malignant lesions in relation to promotion of early carcinogenesis.

PGE2 is important in this process through activation of prostaglandin E receptors (EP1, 2 and 4) that increases β-catenin nuclear accumulation and transcriptional activity (Castellone et al., 2005), and the activation of several signal transduction pathways favoring malignant growth by the enhancement of cellular proliferation, promotion of angiogenesis, inhibition of apoptosis, stimulation of invasion/migration, and suppression of immune responses (Wang and Dubois, 2006; Pai et al., 2002; Kawamori et al., 2003). In addition, PGE2 increases the incidence and multiplicity of tumors in models of chemically induced colon carcinogenesis in rodents treated with the prostanoid (Kawamori et al., 2003).

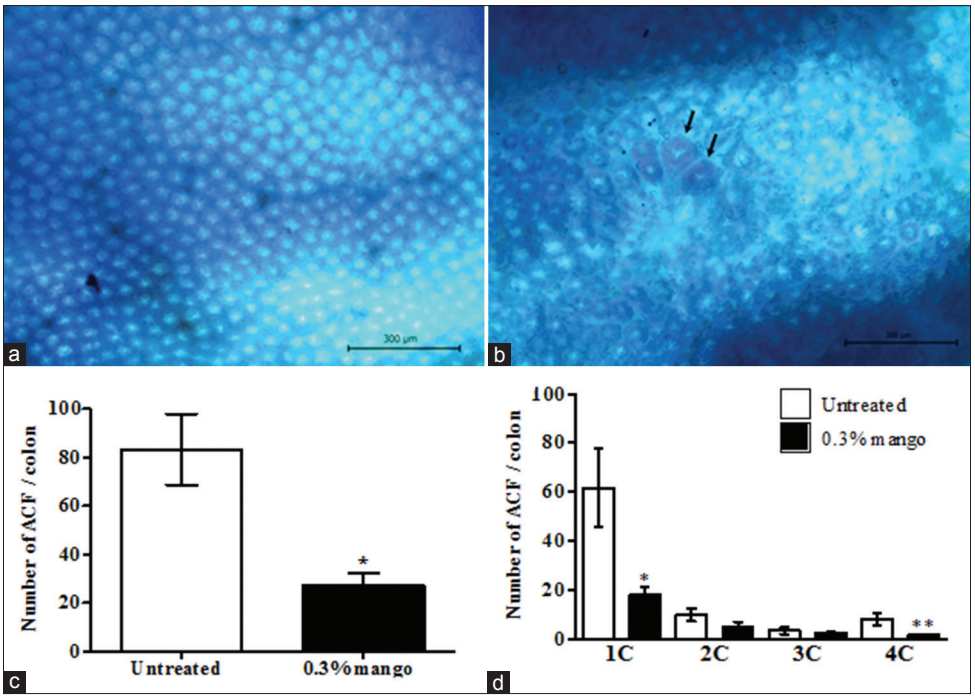


Fig 1. Mango juice reduced significantly the formation of ACF. AOM-treated mice received 0.3% of mango juice or drinking water (control) for eight weeks. (a, b) Representative image of colonic mucosa stained with methylene blue (100X). Arrows indicate ACF localizations (A, 0.3% mango; B, untreated). (c) Number of ACF/colon. (d) Number of ACF/colon. Number of foci containing one (1C), two (2C), three (3C), and four or more (4C+) aberrant hyperproliferative crypts. *p<0.05 mango treated vs untreated; **p<0.01 mango treated vs untreated. Columns, means from six mice in each group; bars, standard error.

In this study, PGE2 levels were significantly reduced by the consumption of WMJ during 8 weeks after AOM injections, compared with the control group (Fig. 2a; p=0.019). The concentration range of PGE2 in control group (AOM, water) was 60 – 1300 pg/mL, but in animals that received WMJ for 8 weeks after AOM treatment, PGE2 concentration was less than 19pg/mL (detection limit of assay). There was also a high correlation between PGE2 levels and ACF, which indicates a possible mechanism for the control of carcinogenesis in colonic mucosa by mango (Fig. 2b).

The carcinogenic role of PGE2 in this pre-clinical model are in concordance with previous findings when deletion or inhibition of PGE2 receptors (EP1 and 4) reduced the induction of ACF by AOM to 67% or less during the early stages of colorectal AOM-induced carcinogenesis (Eisinger et al., 2007). A similar result was observed in mice that developed less and smaller ACF when treated with AOM and when the production of PGE2 was blocked. PGE2 levels in colonic mucosa was significantly induced by AOM injections in control group (AOM, water), which might be the result of IL1β effect as it was also increased in this group (Apte et al., 2006). The contrary happened in experimental group (AOM, mango 0.3%) where regular mango consumption significantly reduced PGE2 levels. These results are in line with earlier reports employing other agents such as silibinin and grape seed extract (Velmurugan et al., 2008, 2010).

Pro-inflammatory cytokines TNFa and IL1(a and b) are considered "alarm cytokines" and tumor promoting factors (Babbar and casero, 2006) by stimulating chemokine expression and reactive oxygen species (ROS) production within epithelial cells (Terzic et al.,2010; Smyth et al.,2004). IL1β causes inflammation and induces the expression of pro-inflammatory genes (Apte et al., 2006).

Here, the levels of pro-inflammatory cytokines in blood between the control and experimental groups were only slightly but not significantly different (Fig. 2c). TNFα levels were below the detection limit of the assay, and IL6 levels were the same in the three groups of animals. A novel result of our work is the increased level of IL1β induced by AOM, which is reported here for first time in early stages of post-initiation of colon carcinogenesis (data no shown), and the trend to diminish IL1β level with WMJ consumption. Previous studies have highlighted the role of IL1β in the development of chemically induced tumors (Krelin et al., 2007), and it has been proposed to be involved in the process of mutagenesis by activation of target cells for transformation to produce mutagens (e.g ROS) and/or stimulation of proliferation of pre-malignant cells (Apte et al., 2006).

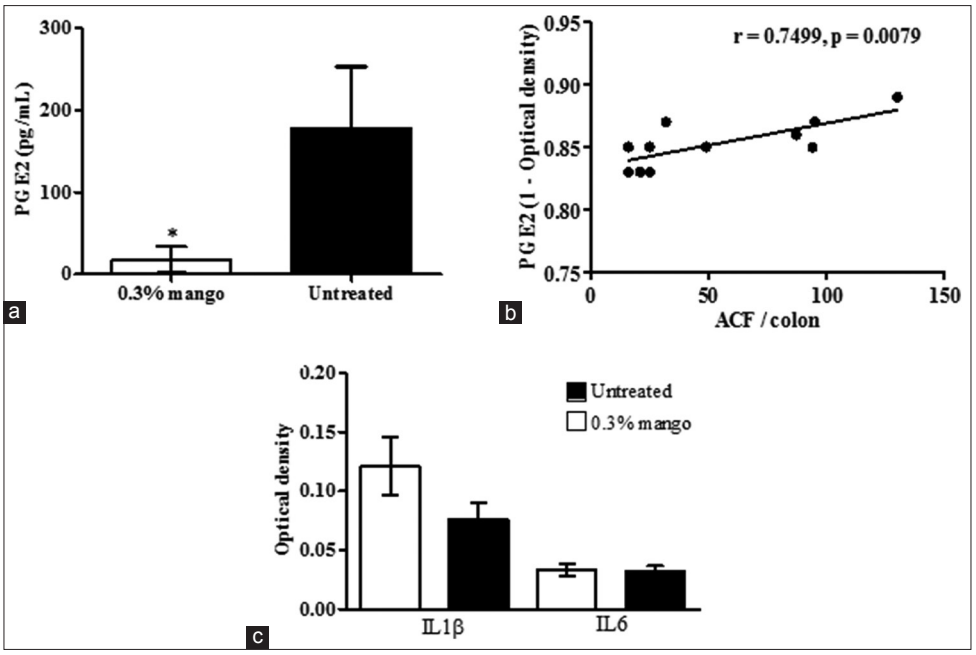


Fig 2. Mango reduces levels of inflammatory biomarkers induced in colon (PGE2) but not in circulation (IL1β, IL6 and TNFα (not shown)) in AOM-treated mice. (a) Levels of PGE2 in colonic mucosa were measured and (b) they were correlated with ACF formation. (c) IL1b and IL-6 blood levels were measured (optical density). Columns, means; bars, standard error. *P<0.05, mango treated vs untreated mice.

The available information of serum levels of cytokines on colon carcinogenesis is scarce and only found in cohort studies which resulting from association between inflammatory cytokines and increased risk of colorectal adenoma (Long and Raufman, 2011). Our data are in agreement with few reports in human beings where it has been encountered a dysregulated expression of inflammatory cytokines and chemokines in the transition from normal mucosa to adenomatous polyp (McLean et al., 2011).

Systemic effects of dietary compounds such as changes of blood levels of cytokines are dependent of bioaccesible fraction of phytochemicals or their metabolites, which are reduced after the digestive process by 0.3 to 53%. Then, the accumulation of bioactive compounds reached in gut of mice who received mango juice in our study (AOM, mango 0.3%) could not be enough to reduce the systemic cytokines levels (Neilson et al., 2011; Cilla et al., 2009; Romier et al., 2009). Nonetheless, it is remarkable that intact or modified phytochemicals from mango were enough to act locally and control carcinogenesis halting ACF formation in the experimental group (AOM, mango 0.3%). It would be interesting to measure protein levels of these cytokines in colon and correlate them with chemopreventive effects of mango.

This early modulation of inflammatory factors during pre-malignant stages of colon carcinogenesis may be because this is a tissue where there is a permanent exchange of regulatory signals between cells via the production of mediators (cytokines, growth factors, adhesion molecules, and neuropeptides) and/or cell events (proliferation and apoptosis), and it is vulnerable to the threat (in the case of AOM) or the protection (in the case of mango) from the surrounding environment (Maldonado-Rojas et al, 2011).

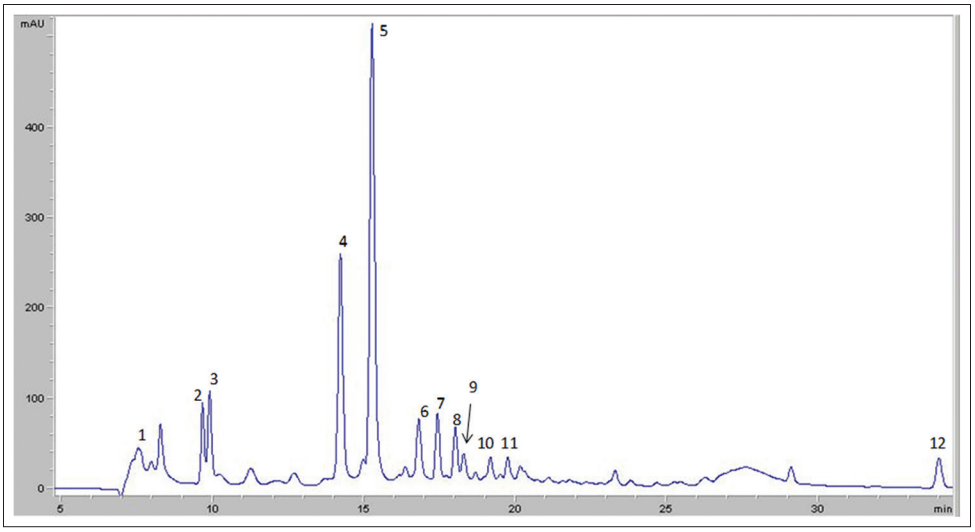
Anti-inflammatory properties of phytochemicals have been described at the level of gene expression of inflammation-related genes (Bousserouel et al., 2011; Kauntz et al., 2012), but it has also been described the interaction of carotenoids, phenolic compounds (gallic acid, quercetin, genistein, apigenin, cyaniding, kaempferol, resveratrol, curcumin) and cycloxygenases enzymes (COX-1/COX-2) leading to the reduction of prostanoid accumulation, particularly PGE2 (Maldonado-Rojas et al., 2011; Mitjavila et al., 2012).

The bioactive compounds frequently found in fruits such as mango, are highly multifunctional and interfere with initiation, promotion and progression of cancer by inhibiting molecular targets that play important roles in both inflammation and colon cancer (Madka and Rao, 2013). These actions are reached in part by the antioxidant capacity of phytochemicals to regulate the activation of redox sensitive molecules involved in colon carcinogenesis. For example the transcription factors NFkB and activator protein 1 (AP-1) which act as molecular switches to turn normal cells into premalignant cells through activation of

genes involved in inflammation and cellular proliferation (Surh et al., 2005).

From 12 peaks shown in the chromatogram obtained after the injection of hydrophilic extract, six were identified as shikimic, chlorogenic (peaks 2 and 12), siringic and gallic acids (peak 6 and 9) when they were compared with standards, and the match was more than 95% (Fig. 3). It indicates that the sample contains these four kinds of phenolic acids, but an additional characterization is needed to find out if they are glycosilated, especially with regard to chlorogenic and gallic acids peaks with different retention times. In the lipophilic extract, β-carotene was the main compound in this sample. In other hand, is important to know the content of mangiferin in this whole mango juice, because is a compound with chemopreventive activity at the initiation phase of AOM-induced colon carcinogenesis in F344 rats treated by short-term (4 weeks) with 0.1% mangiferin in a diet, inhbiting significantly the development of ACF exposed to AOM compared to rats treated only with AOM (64.6 ± 22. vs 108.3 ± 43.0) (Yoshimi et al., 2001; Rajendran et al., 2014). Mangiferin is a xanthone that has been described as the predominant compound of Mango Stem Bark (Nunez Selles et al., 2002), however has been found in the flesh of mango mature-green pulp from cultivars Binjai (31.72 μg/g sample), Nam Dok Mai (13.01 μg/g sample) and Kuinin (12.19 μg/g sample), it was also found in ripe pulp from Uba (12.4 mg/kg dry matter), Haden (2.9 mg/Kg dry matter) and Tommy Atkins (2.2 mg/Kg dry matter), seed kernel Uba cultivar (46.5 mg/Kg dry matter) and mango ripe peel (199 mg/kg dry matter) (Manthey et al., 2009; Sulaiman et al., 2012).

Our results of HPLC-DAD show the presence of the phenolic compounds gallic, chlorogenic and shikimic acids which coincide with those reported by Palafox-Carlos in 'Ataulfo' mango pulp (Palafox-Carlos et al., 2012). According to several authors (Palafox-Carlos et al., 2012; Ma et al., 2011; Masibo and He, 2008), phenolic acids are predominant compounds in mango pulp. Some direct effects of gallic acid from mango extracts indicate cumulative NFkB inhibition which results in both anticarcinogenic and antiinflamatory



Peak	R.T*	UV (nm)	Compound**
1	8.246	240	Shikimic acid
2	9.633	261	Chlorogenic acid
3	9.873	266	Unknown
4	14.193	282	Siringic acid
5	15.233	288	unknown
6	16.78	276	Gallic acid
7	17.393	260, 300	Unknown
8	17.993	264	Unknown
9	18.260	272	Gallic acid
10	19.140	295	Unknown
11	19.713	295, 316	Unknown
12	33.966	261	Chlorogenic acid

*Retention time (min); **Spectral matching with original standards was >95%

Fig 3. Chromatogram of phenolic compounds in mango byl HPLC-DAD (280 nm).

properties (García-Riverra et al., 2011). Among the lipophilic compounds, one of the most predominant was b-carotene which coincides with previous reports (Omelas-Paz et al.,2008). It has the advantage to be kept available in the gastrointestinal tract during all the digestive process and the biological effects not related with pro-vitamin A activity have been attributed to its antioxidant property through deactivation of free radicals and singlet oxygen quenching. (Vitale et al., 2010; Dutta et al., 2004).

Although we could not do a precise identification of all the peaks eluted, the chromatographic separation shows a fruit with diverse polyphenolic compounds which acted together to result in the biological effects observed in this study. Thus, a further more detailed characterization is advised, considering specially mangiferin and the glycosed forms.

CONCLUSIONS

Our results suggest that regular intake of mango pulp is effective to control carcinogenesis through the reduction of ACF formation, by means of blocking hyperproliferation which is correlated with decreasing levels of PGE2. This could be a novel mechanism for mango chemopreventive properties that should be investigated further, especially with regard to whether it is associated with less activation of survival pathways stimulated by PGE2.

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Authors' contributions

Andrea Corrales-Bernal performed the experiments, analyzed the results, and preparing the draft manuscript. Gabriela Jaramillo described animals and treatment proceedings. Elhadi Yahia and Andrea Corrales peformed and analyzed mango composition. Maria Elena Maldonado designed experiments, analyzed results and improved the paper.

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