# ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITIES OF Phenax rugosus (POIR.) WEDD AND Tabebuia chrysanta G. NICHOLSON

ACTIVIDAD ANTIINFLAMATORIA Y ANTIOXIDANTE DE Phenax rugosus (POIR.) WEDD Y Tabebuia chrysanta G. NICHOLSON

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## ABSTRACT

Inflammation is a multifactorial and highly regulated process associated to several chronic and degenerative diseases. Due to the fact that many inflammatory processes are sometimes characterized by oxidative stress, it has been demonstrated that antioxidants are useful as anti-inflammatory agents. *Phenax rugosus* (Poir.) Wedd and *Tabebuia chrysanta G*. Nicholson species are used in the Colombian region called "*Eje Cafetero*" due to their antiinfeccious and immunomodulatory properties. *In vivo* anti-inflammatory and *in vitro* antioxidant properties of methanolic and aqueous extracts of the previously mentioned plant species were evaluated in this research. Two acute inflammation models were used for such purpose: TPA-induced mouse ear oedema and carrageenan-induced rat paw oedema. *In vitro* hydroxyl, peroxyl and superoxide scavenging activity of the extracts was evaluated. The aqueous extract of *Phenax rugosus* was found to be promising due to its anti-inflammatory and antioxidant activities. Results obtained from this research are a support for the ethnopharmacological use of these plant species.

Keywords: Phenax rugosus, Tabebuia chrysantha, reactive oxygen species, inflammation.

## RESUMEN

La inflamación es un proceso multifactorial y altamente regulado que acompaña a una gran variedad de patologías crónicas y degenerativas. Dado que muchos procesos inflamatorios crónicos se acompañan de estrés oxidativo, se ha demostrado que extractos y/o compuestos captadores de radicales libres son eficaces como antiinflamatorios. Las especies *Phenax rugosus* (Poir.) Wedd y *Tabebuia chrysanta* G. Nicholson son empleadas en la región cafetera colombiana y se les atribuyen propiedades antiinflamatorias *in vivo* y antioxidantes *in vitro* de los extractos acuosos y metanólicos de estas dos especies vegetales. Se emplearon dos modelos de inflamación aguda: edema auricular inducido por TPA en ratón y edema plantar inducido por carragenina en ratas. La actividad antioxidante *in vitro* se evaluó frente a los radicales hidroxilo, peroxilo y superóxido. El extracto acuoso de *Phenax rugosus* resultó promisorio por sus actividades antiinflamatoria y antioxidante. Estos resultados contribuyen a dar un soporte científico al uso etnofarmacológico de las dos especies vegetales estudiadas.

Palabras clave: Phenax rugosus, Tabebuia chrysantha, especies de oxígeno reactivas, inflamación.

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## INTRODUCTION

Besides the product offer from the pharmaceutical market, there are many other drug products with anti-inflammatory properties. Medicinal plants are still an alternative to the treatment of pathologies that include an inflammatory process. Moreover, the great Colombian plant biodiversity and the existing ethnopharmacological information motivates the research in the phytopharmacological field with the aim of contributing to the knowledge about our medicinal plants.

An enhanced yield of reactive oxygen species -ROS (i.e. superoxide radical, hydrogen peroxide and hypochlorous acid) by phagocitic cells like polimorphonuclear leucocytes (PMNs) has been demonstrated in the acute inflammatory process (1). The magnification of this process leads to an oxidative stress that injuries the inflammatory focus, which is why the antioxidant agents contribute to inhibit this important phase in the inflammatory process (2).

*Phenax rugosus* (Poir.) Wedd (Urticaceae) known as "esparietaria" and *Tabebuia chrysanta* G. Nicholson (Bignoniaceae) known as "guayacán amarillo, cañahuate" species are used in the *Eje Cafetero* due to its anti-infectious and immunomodulatory properties (3-5). The antiinflamatory activity of other species of the *Tabebuia* genus has been demonstrated by means of *in vivo* and *in vitro* studies (6). Lactone-like and lignin-like compounds with *in vitro* activity against HIV have been found in *Phenax rugosus* (Poir.) Wedd (7).

The anti-inflammatory and antioxidant activities of two extracts of *Phenax rugosus* (Poir.) Wedd and *Tabebuia chrysanta* G. Nicholson were evaluated in this research to contribute to the knowledge of their pharmacological properties.

## MATERIALS AND METHODS

### Plants

*Phenax rugosus* was collected at 1,400 m.a.s.l. and at an average temperature of 22°C in the rural area surrounding Cartago, Valle del Cauca, Colombia. *Tabebuia chrysantha was* collected at 1,800 m.a.s.l. and at an average temperature of 20°C in the rural area surrounding Palestina, Caldas, Colombia. Samples of both species were identified and classified at the herbarium of the Faculty of Agricultural Sciences in the Universidad de Caldas. Then, *Phenax rugosus*  (Poir.) Wedd and *Tabebuia chrysantha* G. Nicholson samples were properly compared to the reference samples 1016 and 1525 respectively.

#### Extracts

Leaves were washed with abundant water, and then they were dried at 40°C for 24 hours. Dried leaves were powdered and stored in dark glass containers at -4°C. Aqueous and methanolic extracts were obtained from each plant by maceration. The methanolic extract was rotaevaporated, while the aqueous extract was freeze dried. Extracts were identified as follows: PA (Aqueous extract of *Phenax rugosus*), PM (Methanolic extract of *Phenax rugosus*), TA (Aqueous extract of *Tabebuia chrysantha*) and TM (Methanolic extract of *Tabebuia chrysantha*).

## Phytochemical evaluation

Extracts were analyzed using common chemical tests in order to detect secondary metabolites. Alkaloids were detected with Dragendorff, Valser, Mayer and Reineckatto precipitation reagents. Flavonoids were evaluated by means of the Shinoda test; steroids and triterpenoids through the Liebermann-Burchard reaction; tannins by means of a gelatine-salt reagent; saponins through the haemolysis test; and coumarins by means of a ferric hydroxamate reaction (8).

#### Inflammation tests

#### Animals

Male Wistar rats (10-12 weeks old) weighing about 200 g were used for paw oedema and the ear oedema was performed on male Swiss ICR mice (11-13-weeks old) weighing 34-40 g. The animals were grown and housed at the Pharmacy Department under the following controlled conditions: 12 h light/darkness cycles, 21°C of temperature, water and food *ad libitum*. All experiments were performed according to the ethical considerations of animal care and use (9).

## Paw oedema

As we have proceeded in previous studies (10), the inflammation response was induced by injecting 0.05 mL of carrageenan  $\lambda$  (Sigma) (3% w/v in physiological solution) into the subplantar region in the right hind paw. Thirty minutes before giving the carrageenan injection to the animals, plant extracts (500 mg/kg) and indomethacine (10 mg/kg, as a positive control) were given to them through i.p. route The control group was given saline solution (1 mL/ 100 g). Each group consisted of six animals. The volume of the paws was measured with an Ugo Basile<sup>®</sup> plethysmometer 1, 3 and 5 hours after injecting the carrageenan solution. The antiinflammatory activity was expressed according to the following equation:

$$%I = 100 - \left(\frac{\Delta V_t}{\Delta V_c}\right) \times 100$$
 Equation 1.

Where,  $\Delta V_t$ : delta of the paws' volume in the treated groups, and  $\Delta V_c$ : delta of the paws' volume in the control group.

#### Ear oedema

The TPA-induced oedema was performed on mice by methods commonly used in our research group (11). Briefly, 2.5 µg of phorbol 12-myristate 13-acetate (TPA, Sigma) were dissolved in 20 µL of acetone and, then, topically applied on the right ear of each mouse (n = 6 mice per group). Extracts (1mg/ear) and indomethacine (1mg/ear) were dissolved in acetone and topically applied on the right ear of mice immediately before the TPA was administered. The control group was treated with acetone on the right ear. Four hours after the inflammatory agent was administered, the animals were sacrificed by cervical dislocation. Circular sections (7-mm diameter) were cut from right and left ears. The sections were weighted and then antiinflammatory activity was expressed by means of the following equation:

$$\% I = 100 - \left(\frac{\Delta W_t}{\Delta W_c} \times 100\right)$$
 Equation 2

Where,  $\Delta Wt$ : delta of the ears' weight in the treated groups, and  $\Delta W_{c}$ : delta of the ears' weight in the control group.

#### Antioxidant activity

#### Hydroxyl radical scavenging

The  $\cdot$ OH scavenging activity was evaluated by deoxyribose assay, where the hydroxyl radical is generated by means of a simulation of Fenton reaction (12, 13).

The scavenging test was carried out according to Soobrattee et al. with minor modifications (14). The reagents were added in the following order: 10 µL of plant extracts (10 mg/mL), 100  $\mu$ L of deoxyribose (Sigma, 28 mM), 10 µL of ascorbic acid (Sigma, 5 mM), 780  $\mu$ L of buffer KH<sub>2</sub>PO<sub>4</sub>-KOH (pH 7.4, 10 mM), 50  $\mu$ L of FeCl<sub>3</sub> (400  $\mu$ M in EDTANa<sub>2</sub> 400  $\mu$ M), 50  $\mu$ L of H<sub>2</sub>O<sub>2</sub> (Merck, 28.4 mm). Dimethyl sulfoxide (Sigma, 2 M) was used as reference scavenger. Reaction mixtures were incubated at 37°C for 60 minutes, followed by the addition of 1mL of trichloroacetic acid (Merk®) at 2.8% and 1mL of thiobarbituric acid (Sigma<sup>®</sup>) at 1%. The mixtures were immediately incubated at 92°C for 15 minutes, and then they were cooled down in an ice bath for 5 minutes. The absorbance was measured at 532 nm. The same procedure was made in the series of blanks, but FeCl<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> were avoided. Extracts were dissolved in methanol. Results were expressed as the percentage of the hydroxyl radical scavenging.

In order to dismiss a possible pro-oxidizating effect of the extracts, a control experiment was carried out simultaneously, using the same procedure but without ascorbic acid.

#### Peroxyl radical scavenging

The ABAP/Lysozyme method was used, in which the thermal decomposition of the "azo-starter" 2,2′-azobis(2 methyl-amidine propane) (ABAP) produces peroxyl radical (15).

The reagents were added in the following order: 10  $\mu$ L of the extract (10 mg/mL) or propylgallate (Sigma<sup>®</sup>, 10 mM), 900  $\mu$ L of lysozyme (Sigma<sup>®</sup>, 55.500 U/mL), 100  $\mu$ L of ABAP (Sigma<sup>®</sup>, 100 mM). The reaction mixture was incubated at 45°C for 90 minutes and then cooled down in an ice bath. 50  $\mu$ L of this reaction mixture were taken and added to 950  $\mu$ L of a *Micrococcus lysodeikticus* (Sigma<sup>®</sup>) suspension (0.6 mg/mL dissolved in Dulbecco's buffer). The absorbance change was immediately measured at 450 nm, every 10 seconds for 30 seconds. Results were expressed in terms of percentages of inhibition of the lysozyme. Extracts were dissolved in methanol, and the other reagents were dissolved in phosphate buffer 50 mM pH 7.4.

#### Superoxide radical scavenging

The superoxide radical was generated by the action of the xanthine oxidase on the hypoxanthine. It produced a NBT reduction (nitroblue tetrazolium), generating a diformazan compound (12).

Reagents were added in the following order: 10  $\mu$ L of extracts (10mg/mL) or n-propylgallate (Sigma<sup>®</sup>, 10 mM/mL), 100  $\mu$ L of EDTANa<sub>2</sub> (Sigma<sup>®</sup>, 10 mM), 10  $\mu$ L of hypoxanthine (Sigma<sup>®</sup>, 10 mM), 100  $\mu$ L of NBT (Sigma<sup>®</sup>, 1 mM), and phosphate buffer pH 7.4 (50 mM) until completing 1.2 mL of reaction mixture. Then, 100  $\mu$ L of xanthine oxidase (Sigma<sup>®</sup>, 0.66 U/mL) were immediately added, and the absorbance change was measured every 10 seconds for 2 minutes at 560 nm.

## Statistical analysis

For each test, results were expressed as the mean of the values  $\pm$  the standard deviation. The obtained data were subjected to an analysis of simple variance (ANOVA), followed by the analysis of multiple comparisons (Tukey's test). Suppositions of normality, homogeneity, and independence of variances were previously verified.

## **RESULTS AND DISCUSSION**

#### Composition of secondary metabolites

By preliminary phytochemical analysis of the extracts, positive test was obtained for: PA: Saponins, coumarins and/or terpenic lactones; PM: Saponins, coumarins and/or terpenic lactones, steroids and/or triterpenoids; TA: Alkaloids, tannins, saponins, coumarins and/or terpenic lactones; TM: Alkaloids, tannins, saponins, coumarins, coumarins and/or terpenic lactones, steroids and/or triterpenoids. These data coincide with the data reported for other species of *Tabebuia* genus (16-18).

#### Anti-inflammatory effect

TPA induced ear oedema is useful for the *in vivo* evaluation of possible cyclooxygenase pathway inhibitors, due to the fact that TPA induces the liberation of arachidonic acid, increasing PGE<sub>2</sub> production in the inflammatory focus.

In table 1, it is shown that all the evaluated extracts presented a significant anti-inflammatory effect in the TPA induced ear oedema, with inhibition levels from 17% to 32%. The PM extract showed a better anti-inflammatory effect. Methanolic extracts have steroids and/or triterpenoids, which increase the lypophilic character of the PM extract. The possible active compounds can be better absorbed through the skin of the ear of the animal. This fact would allow a better anti-inflammatory effect that the one presented by aqueous extracts. Furthermore, it is important to say that aqueous extracts are not dissolved appropriately in the vehicle commonly used in this test, which is why it is difficult to detect their possible anti-inflammatory effect in this animal assay.

**Table 1.** Anti-inflammatory effect of *Phenax rugosus*(Poir.) Wedd and *Tabebuia chrysantha* G. Nicholson inTPA induced ear oedema.

Group	$\Delta$ weight (mg)	% Inhibition
CONTROL	22.5±3.7	
INDO	12.4± 2.1**	44.8±9.2
PA	18.6± 1.1**	$17.4 \pm 4.8$
PM	$15.3 \pm 2.0^{**}$	31.8±9.1
TA	17.2 ±1.8**	23.8±8.0
ТМ	17.9 ±1.2**	20.6±5.5

INDO (Indomethacine), PA (*Phenax rugosus* aqueous extract), PM (*Phenax rugosus* methanolic extract), TA (*Tabebuia chrysantha* aqueous extract), TM (*Tabebuia chrysantha* methanolic extract). Mean  $\pm$  E. D. (n=11 mice per group), \*\* p< 0.01 against control group (Tukey's test).

The paw oedema induced by the carrageenan is a two-phase inflammation model. The initial phase is characterized by the liberation of mediators like bradykinin, which produces a maximum of inflammation at around the third hour. In the second phase, important quantities of prostanglandins and lytic enzymes are released. Most of AINEs-like anti-inflammatory agents can be detected in this last phase (19).

Carrageenan induced paw oedema is an useful model to evaluate compounds with anti-inflammatory activity when they are administered by oral or parenteral route (i.e intraperitoneal route). As it is shown in table 2, all extracts significantly inhibit the development of the inflammation after three hours of carrageenan administration. The TA extract is able to inhibit the oedema from the first hour. This early effect is not common in vegetable extracts, and it could be related with a possible differential mechanism against the other evaluated extracts. As it was previously mentioned, the initial phase is influenced by the release of vasoactives mediators like kinines, histamine and serotonine. Also, in this early phase, there are important leukocyte migrations toward the inflammatory focus (19). This fact suggests that the TA extract participates in mechanisms related to the initial mediators of the acute inflammation, besides a possible AINE-like mechanism shared with the other extracts. This observation should be approached in mechanistic studies. The anti-inflammatory activity of other *Tabebuia* species has been evaluated *in vivo* and *in vitro* tests. These studies suggest mechanisms related to arachidonic acid pathway inhibition (6, 20). For the *Phenax rugosus* species, an anti-inflammatory effect has been reported on the oedema produced by the bite of the *Bothrops asper* snake (21).

**Table 2.** Antiinflammatory effect of *Phenax rugosus* (Poir.) Wedd y *Tabebuia chrysantha* G. Nicholson in paw oedema induced by carrageenan.

	Hour 1		Hour 3		Hour 5	
Group	$\Delta \operatorname{vol}$	Inhibi-	$\Delta$ Vol.	Inhibi-	$\Delta$ Vol.	Inhibi-
	$(mL \ge 10^2)$	tion %	$(mL \ge 10^2)$	tion %	$(mL \ge 10^2)$	tion %
CON- TROL	25±6		77±24		100±17	
INDO	13±4**	49±16	18±17**	77±4	30±6**	70±6
PA	19±3	24±10	23±25**	70±7	26±6**	74±6
PM	18±3	27±10	39±24**	49±12	45±9**	55±9
TA	$14 \pm 4^{**}$	45±14	20±41**	74±11	23±7**	77±7
ТМ	19±2	24±8	25±18**	65±6	30±4**	70±4

INDO (Indomethacine), PA (*Phenax rugosus* aqueous extract), PM (*Phenax rugosus* methanolic extract), TA (*Tabebuia chrysantha* aqueous extract), TM (*Tabebuia chrysantha* methanolic extract). Mean  $\pm$  E. D. (n=6 rats per group), \*\* p< 0.01 against control group (Tukey's test).

The anti-inflammatory activity found in these plants coincides with the activity reported for other species with similar secondary metabolites. Recent studies have demonstrated the anti-inflammatory activity of compounds such as saponins, tannins, flavonoids, and alkaloids (22-24).

When these same extracts were administered via gavage (data not shown), they didn't show any antiinflammatory effect on the paw oedema induced by carrageenan. It is possible that the metabolism of first-step and other pre-systemic effects doesn't allow appropriate plasmatic levels of the extract's anti-inflammatory compounds.

#### Antioxidant activity

The antioxidant activity against hydroxyl radical was evaluated by deoxyribose assay. In this reaction, the reduced iron (by the ascorbate presence) reacts to hydrogen peroxide producing the •OH. This radical promotes the degradation from deoxyribose to malonyldialdehyde, which can be detected by addying thiobarbituric acid; this addition produces a pink cromophore (12). In table 3, it is shown that all extracts possess a significant scavenging effect of the hydroxyl radical (between 57% and 86%). In a parallel experiment in absence of ascorbate (data not shown), it was detected that the TA and TM extracts could also mediate through a reducing effect the production of hydroxyl radical. In spite of this effect these extracts possess a major hydroxyl scavenging effect. The PA extract presents less interference in this sense, and it presents an excellent scavenging activity of the hydroxyl radical (86%). It is very important to find scavenging agents for the hydroxyl radical, because there are no antioxidant enzymatic systems that attenuate the effect of this radical on oxidative stress processes (13).

**Table 3.** *In vitro* scavenging activity of hydroxyl radical of *Phenax rugosus* (Poir.) Wedd and *Tabebuia chrysanta* G. Nicholson. Deoxiribose assay.

Sample	Absorbance x 1000	Scavenging of hydroxyl radical (%)
CONTROL	100±16	0
DMSO	21±3**	79±3
PA	14±3**	86±3
PM	43±7**	57±7
ТА	17±1**	83±1
ТМ	20±3**	80±3

DMSO (Dimethilsulfoxide), PA (*Phenax rugosus* aqueous extract), PM (*Phenax rugosus* methanolic extract), TA (*Tabebuia chrysantha* aqueous extract, TM (*Tabebuia chrysantha* methanolic extract). Mean  $\pm$  E. D. (n=4), \*\* p< 0.01 against control group (Tukey's test).

The peroxyl radical inhibits the lysozyme enzyme and, depending on the damage caused to this enzyme, it would be possible to know if a compound does or does not scavenge these radicals. Thus, the residuary lysozyme activity on *Micrococcus lysodeikticus* is an indicative of the damage of the enzyme by peroxyl radical (12, 25).

In table 4, data shows that only the PA extract protects lysozyme from the damage by the peroxyl radical. In a parallel experiment (data not shown), we found that the enzymatic activity of the lysozyme decreases 20% in presence of the PA extract. This apparent inhibitory effect is not very important for the scavenging assay, it only presents a light interference like false negative when interpreting the antioxidant capacity of the PA extract. **Table 4.** *In vitro* scavenging activity of the peroxyl radical of *Phenax rugosus* (Poir.) Wedd and *Tabebuia chrysanta* G. Nicholson. ABAP/Lysozyme method.

Sample	Delta of absorbance x 1000	Lysozyme inhibition (%)
CONTROL	208±44	0
Methanol	142±26*	32±13
n-propilgallate	185±19	15±4
PA	181±8	13±4
PM	90±24**	57±11
ТА	138±16*	33±8
ТМ	77±14**	63±7

PA (*Phenax rugosus* aqueous extract), PM (*Phenax rugosus* methanolic extract), TA (*Tabebuia chrysantha* aqueous extract, TM (*Tabebuia chrysantha* methanolic extract). Mean  $\pm$  E. D. (n=4), \*p< 0.05 y \*\*p<0.01 against control (Tukey's test).

In the generation / scavenging test of the superoxide radical, the scavengers of this radical inhibit the NBT reduction. The test evaluates the possible interference of extracts on the xanthine oxidase, this enzyme generates the superoxide radical (12).

In a control experiment (data not shown), it was demonstrated that the evaluated extracts do not inhibit the xanthine oxidase activity. In table 5, it is shown that all extracts were able to scavenge the superoxide radical more than 50%, specially the TM extract. In spite of superoxide dismutase constituting the first line of the antioxidant defence system in situations of oxidative stress, it is advantageous to have additional scavenging agents such as vegetable extracts. It is worth taking into account the importance of scavenging the superoxide radical, because this radical is the first ROS that takes place in the molecular oxygen reduction cascade. Thus, this radical is the source for the formation of other species that also participate in oxidative stress processes (26).

**Table 5.** *In vitro* scavenging activity of superoxide radical of *Phenax rugosus* (Poir.) Wedd and *Tabebuia chrysanta* G. Nicholson. Xanthine /Xanthine Oxidase method.

Sample	Delta of Absorbance x 1000	Scavenging of superoxide radical (%)
CONTROL	146±69	0
n-propilgallate	18±7**	87±1
PA	65±27**	56±3
PM	58±24**	61±3
TA	$60 \pm 27^{**}$	59±2
ТМ	72±28**	71±6

PA (*Phenax rugosus* aqueous extract), PM (*Phenax rugosus* methanolic extract), TA (*Tabebuia chrysantha* aqueous extract, TM (*Tabebuia chrysantha* methanolic extract). Mean  $\pm$  E. D. (n=4), \*\* p< 0.01 against control group (Tukey's test).

In summary, the four evaluated extracts present scavenging activity at least against one of the free radicals evaluated. Aqueous extracts present a better scavenging activity than methanolic extracts. The PA extract also presents a better anti-inflammatory effect in the carrageenan induced paw oedema. As it has been previously demonstrated, inflammation is characterized by the activation of phagocytic cells that synthesize and liberate a great quantity of ROS (26). For this reason, scavenging a free radical represents a viable anti-inflammatory action mechanism for a large quantity of compounds (27), especially the compounds from natural sources. This fact has been demonstrated for several vegetable species (28-31).

## CONCLUSIONS

In conclusion, aqueous and methanolic extracts of the *Phenax rugosus* (Poir.) Wedd and *Tabebuia chrysantha* G. Nicholson species present *in vivo* anti-inflammatory activity and *in vitro* antioxidant activity. Further studies are required for researching on the anti-inflammatory action mechanisms and elaborating on the profile of the antioxidant activity in cases of *in vivo* oxidative stress. The results of this research contributed to the pharmacological knowledge of the two vegetable species studied.

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## GRUPO DE EXTENSIÓN SOLIDARIA E INVESTIGACIÓN EN SEGURIDAD ALIMENTARIA PARA LA REGIÓN VICERRECTORÍA DE EXTENSIÓN

## Facultad de Química Farmacéutica

Departamento de Alimentos Universidad de Antioquia



- Asesoría técnica en procesos de alimentos a microempresas y vendedores ambulantes.
- Propuesta educativa para el fomento de hábitos de alimentación saludable en estudiantes de básica primaria y bachillerato (Media Técnica).
- Asesorías para comedores comunitarios y restaurantes escolares con énfasis en transformación de alimentos, apun-

tando a mejorar el consumo y la calidad de los nutrientes carenciales que padece nuestra población.

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- Formulación y ejecución de proyectos comunitarios enfocados a la explotación de recursos agrícolas de la región.
- Capacitación en transformación de alimentos tales como: Yogur, Kumis, Queso Crema, Mermeladas, Compotas, Pulpas, entre otros.
- Asesoría empresarial en las buenas prácticas de manufactura.

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