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The Relationship of *In Vitro* Anti-Inflammatory and Antioxidant Activity with Total Phenolic and Flavonoids Content of *Tithonia diversifolia* Leaves Ethanolic Extract and Fractions

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Abstract
A previous study showed that *Tithonia diversifolia* ethanolic extract has antiproliferative properties against HeLa cervical cancer cells. It has also shown anti-inflammatory properties *in vivo*, one of the approaches to cancer chemoprevention. This research aimed to determine the relation of *in vitro* anti-inflammatory and antioxidant activity of *T. diversifolia* leaves ethanolic extract and fractions with total phenolic and flavonoid content. The anti-inflammatory was evaluated *in vitro* using red blood cell (RBC) membrane stabilization and inhibition of protein denaturation method, while antioxidant activity was done using the DPPH method. Phytochemical screening was done using the TLC method. Total phenolic and flavonoid content were also counted using the colorimetry method. ANOVA followed by LSD post hoc, multiple regression analysis, and Pearson correlation were used to analyze the data. Compared to its fractions, the highest anti-inflammatory and antioxidant activity was obtained from *T. diversifolia* leaves ethanolic extract. The *T. diversifolia* leaves ethanolic extract contained terpenoids, alkaloids, polyphenols, and flavonoids. Overall, the antioxidant property contributed more to RBC membrane stabilization than protein denaturation inhibition. The phenolic and flavonoid content contributed most to antioxidant and red blood cell membrane stabilization rather than protein denaturation inhibition.

Keywords: Anti-inflammatory; Antioxidant; Phytochemical screening; *Tithonia diversifolia* leaves extract and fractions; Total phenolic and flavonoids content

1. INTRODUCTION

Inflammation is a defense mechanism to local injury that protects from tissue damage caused by physical, chemical, or microbiological trauma (Tu et al., 2022). Inflammation is the cause of many diseases, including cancer (Greten & Grivennikov, 2019). The incidence of cancer in the world is increasing. According to estimates (Siegel et al., 2023), there were 609,820 cancer deaths and 1,958,310 new cancer cases in the US alone. Cancer is the cause of one death out of every six (World Health Organization, 2022). An approach to cancer chemoprevention is anti-inflammatory drugs (Greten & Grivennikov, 2019). First-line anti-inflammatory drugs can cause serious side effects, e.g., impaired renal function, hypertension, edema, gastrointestinal bleeding, and even increase the risk of heart failure, stroke, myocardial

infarction, and death (Bindu et al., 2020). To avoid these side effects caused by anti-inflammatory drugs, researchers are exploring new anti-inflammatory candidates, including those from natural products, e.g., plants (Nunes et al., 2020). One candidate that could be explored as an anti-inflammatory agent is *Tithonia diversifolia* (Hemsl) A. Gray.

A previous study showed that ethanolic extract of *T. diversifolia* leaves exhibited antiproliferative activity on HeLa cervical cancer cells (Puspitasari et al., 2022). *T. diversifolia* is used as an anti-inflammatory preparation by society (Dalimartha, 1999). Ethanolic extract of *T. diversifolia* leaves exhibited anti-inflammatory activity *in vivo* by reducing the exudate volume of carrageenan-induced mice paw edema models (Lubis et al., 2020). The polar extract of *T. diversifolia* leaves contains chlorogenic acid and sesquiterpene lactone, which show better anti-inflammatory effects than indomethacin (Lopes et al., 2021). *T. diversifolia* leaves exhibit antioxidant activity (Tamfu et al., 2022). Compounds with antioxidant activity usually have anti-inflammatory properties (Bourais et al., 2023). This research was conducted to reveal a detailed mechanism of action of the anti-inflammatory properties of *T. diversifolia* leaves ethanolic extract and their fractions, i.e., n-hexane, dichloromethane, and methanol-water fraction, their antioxidant properties, as well as to determine the relationship of anti-inflammatory and antioxidant activity with total phenolic and flavonoids content. Fractionation of an extract is a further step for natural products' isolation (Xin et al., 2021). Thus, this research was aimed at preliminary bioactivity-guided isolation for the anti-inflammatory and antioxidant activity of *T. diversifolia* leaves ethanolic extract.

2. MATERIAL AND METHODS

2.1. Extraction and fractionation of *T. diversifolia* leaves

T. diversifolia leaves were collected from Jember. They were hand-picked based on healthy and mature leaves and taken from a mature plant starting from the 5th strand from the tip of the stem to the base. The plant identity was confirmed using plant determination at the Botany Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences University of Jember. The leaves were air-dried, pulverized, and sifted prior to extraction. The extraction used maceration on 100-gram leaves with continuous stirring with 96% ethanol as solvent (1:7 ratio). The maceration was repeated three times. The extract was evaporated using a rotary evaporator (Heidolph Laborota 4000) until a thick extract was obtained (Puspitasari et al., 2022). A 20-gram thick ethanolic extract was then diluted with methanol-water (1:1), followed by fractionation using the liquid-liquid partition method with n-hexane, dichloromethane, and methanol-water using a 1:1 ratio. The fractions were evaporated using a rotary evaporator until thick fractions were obtained.

2.2. Anti-inflammatory assay using red blood cells membrane stabilization method

Ethical clearance was obtained from KEPK FKG Jember University with number 1423/UN25.8/KEPK/DL/2021. The red blood cell (RBC) membrane stabilization method was done based on (Yesmin et al., 2020) with slight modification. RBCs were obtained from female rabbits, placed in EDTA tubes, and centrifuged at 3,000 rpm for 15 min at room temperature.

The residue was washed with isosaline until the supernatant was clear. The RBC obtained was suspended 10% in isosaline.

All of the experiments were carried out in three replicates. A test solution of 4.5 mL was prepared by adding 1 mL of 0.15 M PBS pH 7.4 with 0.5 mL of RBC suspension (2% v/v), 1 mL of sample solution with the series concentration of 500, 250, 125, 62.5, and 31.3 µg/mL, and 2 mL of hyposaline solution (0.25% b/v). Sodium diclofenac (Phapros, series concentration of 500, 250, 125, 62.5, and 31.3 µg/mL) was used as a positive control. The mixture was incubated at 56 °C for 30 min in a water bath, followed by centrifugation at 5,000 rpm for 10 min at room temperature, and read at 560 nm absorbance using an ELISA reader (Dialab EL X800). The stability of red blood cells was then calculated based on Formula 1.

$$\text{Red blood cell membrane stability (\%)} = \frac{(100-(\text{sample absorbance}-\text{control absorbance}))}{(\text{control absorbance})} \text{ x } 100\%$$

Formula 1. The calculation of the percentage stability of red blood cell membrane.

The plot of concentration vs. RBC membrane stability was made as the basis of the IC_{50} calculation.

2.3. Anti-inflammatory assay using protein denaturation inhibition method

The protocol used for this method was adapted from Saleem et al. (2020). The reaction mixture consisted of 0.2 mL of 1% bovine serum albumin (BSA, BioPLUS) and 2.8 mL of PBS (pH adjusted to 6.4 with 1N HCl) to which normal saline (negative control), test extract/fractions (500, 250, 125, 62.5, and 31.3 µg/mL), or sodium diclofenac (500, 250, 125, 62.5, and 31.3 µg/mL) were added as positive controls. The mixture was incubated at 37 ºC for 20 minutes and heated to 51 ºC for 20 minutes. After cooling, the absorbance was measured at 660 nm. The experiment was carried out in three replicates. Inhibition of protein denaturation was calculated based on Formula 2.

$$\text{Protein denaturation inhibition (\%)} = \frac{(\text{control absorbance}-\text{samp absorbance})}{(\text{control absorbance})} \times 100\%$$

Formula 2. The calculation of the percentage protein denaturation.

The plot of concentration vs protein denaturation inhibition was made as the basis of the IC_{50} calculation.

2.4. Antioxidant assay using DPPH method

The antioxidant assay was done using the DPPH microplate assay. Briefly, the extract and fractions were diluted using methanol (Merck), and then 150 µl of the sample was placed in a 96-well plate with 100 µl of 0.2 mM DPPH solution (Sigma Aldrich). After incubation for 30 minutes at room temperature in a dark room, the absorbance was measured at 515 nm using an ELISA reader (Dialab EL X800). Vitamin C (Emsure, concentration of 21.2, 10.6, 5.3, 2.65, 1.325, 0.663, 0.331, and 0.116 µg/mL) was used as a positive control. The experiment was carried out in three replicates. A series of concentrations used ranging from 100.6-0.686 µg/mL for the ethanolic extract, 500-3.906 µg/mL for n-hexane fraction, 106-0.828 µg/mL for

dichloromethane fraction, and 104-0.813 µg/mL for methanol-water fraction, to make sure that the IC_{50} calculated by interpolation of each sample. The antioxidant activity was calculated based on Formula 3.

$$\text{DPPH scavenging activity (\%)} = \frac{(\text{control absorbance} - \text{sample absorbance})}{(\text{control absorbance})} \times 100\%$$

Formula 3. The calculation of percentage antioxidant activity by DPPH scavenging activity.

The plot of concentration vs DPPH scavenging activity was made as the basis of the IC_{50} calculation (Nasution et al., 2019).

2.5. Phytochemical screening

The phytochemical screening of the extract and fractions of *T. diversifolia* leaves was done based on the TLC method with the respective chemical spray to detect terpenoids (anisaldehyde sulfuric acid), alkaloids (Dragendorff's reagent), polyphenols ($FeCl_3$), and flavonoids (boric citrate) (Moulishankar, 2021). The TLC was done using silica gel 60 F_{254} as the stationary phase. In contrast, the mobile phase was the optimum composition, resulting in the best separation and resolution for the spots found in each sample. The mobile phase used was and n-hexane (Merck): ethyl acetate (Merck) (3:2) for ethanol extract, toluene (Merck): ethyl acetate (Merck) (7:3) for n-hexane fraction, n-hexane (Merck): ethyl acetate (Merck) (2:1) for dichloromethane fraction, and n-hexane (Merck): ethyl acetate (Merck): methanol (Merck) (1:1:2) for methanol-water fraction.

2.6 Total phenolic content

The experiment was carried out in three replicates. A 100 µl sample (extract or fractions at the concentration of 1,060 µg/mL (ethanolic extract); 1,010 µg/mL (n-hexane fraction); 1,040 µg/mL (dichloromethane fraction); and 1,000 µg/mL (methanol-water fraction)) solution in methanol (Merck) was reacted with 100 µl Folin Ciocalteu reagent (Merck) in 96 healthy plate. A 50 µl of Na_2CO_3 (Merck) was added. After 30 min of incubation at room temperature in a dark place, the absorbance was measured at 735 nm using an ELISA reader (Dialab EL X800). The phenol content was calculated by plotting the sample absorbance into the standard curve of gallic acid (Sigma Aldrich, concentration of 50, 40, 30, 20, and 10 µg/mL) (Loucif et al., 2020).

2.7. Total flavonoid content

The experiment was carried out in three replicates. Sample (extract or fractions at the concentration of 1,060 µg/mL (ethanolic extract); 1,010 µg/mL (n-hexane fraction); 1,040 µg/mL (dichloromethane fraction); and 1,000 µg/mL (methanol-water fraction)) was dissolved in methanol and pipetted 100 µl in a 96 well plate, followed by the addition of 100 µl $AlCl_3$ 50 µl CH_3COOK 10%, and 50 µl methanol (Merck). The incubation was done for 30 min in a dark room at room temperature. Then, the absorbance was measured at 425 nm using an ELISA reader (Dialab EL X800). The flavonoid content was calculated as quercetin equivalent based on a quercetin (Sigma Aldrich) standard curve plot with concentrations of 40, 30, 25, 20, and

10 µg/mL (Loucif et al., 2020).

2.8. Data analysis

The IC_{50} obtained from the anti-inflammatory and antioxidant assay and the total phenolic and flavonoid content were evaluated for their differences using ANOVA, followed by LSD using a 99% confidence level. The multiple regression analysis was done to analyze whether total phenolic and flavonoid content contributed to the anti-inflammatory and antioxidant activity, while the Pearson correlation analysis was done to determine the correlation between parameters in this research.

3. RESULTS AND DISCUSSION

Plant determination has established that the plant's identity was *T. diversifolia*. The extract and fraction yields obtained are shown in Table 1. The yield difference was suggested due to the different solubility of the compounds fractionated by different solvent polarities. The highest yield was obtained from the methanol-water fraction, suggesting that the ethanolic extract of *T. diversifolia* leaves contained more polar compounds than non-polar and semi-polar compounds.

The anti-inflammatory activity of each sample is shown in Table 2. Even though the anti-inflammatory activity of the extract and fractions was incomparable to sodium diclofenac as the positive control for these assays, the IC_{50} for the extract and fractions was considered promising. All the samples exhibited significantly different anti-inflammatory in both methods. The ethanolic extract of *T. diversifolia* leaves showed the highest anti-inflammatory activity on both methods.

Table 1. Extract and fractions yield obtained from *T. diversifolia* leaves.

Sample	Yield (%)
Ethanolic extract of *T. Diversifolia* leaves	23.425
N-hexane fraction of *T. Diversifolia* leaves	10.217
Dichloromethane fraction of *T. Diversifolia* leaves	22.751
Methanol-water fraction of *T. Diversifolia* leaves	49.215

The antioxidant activity of *T. diversifolia* leaves ethanolic extract and its fractions are shown in Table 3. The vitamin C as a positive control showed that the method had been carried out correctly. The IC_{50} values of all samples were significantly different. Both ethanolic extract and methanol-water fraction of *T. diversifolia* leaves showed strong antioxidant activity, but the other two (n-hexane and dichloromethane fraction of *T. diversifolia* leaves) exhibited intermediate antioxidant activity. Again, the ethanolic extract of *T. diversifolia* leaves showed the highest antioxidant activity.

The phytochemical screening showed that the ethanolic extract of *T. diversifolia* leaves contains more complex compounds compared to its fractions (Figure 1 and Table 4). The fractionation did separate the compounds based on their polarity. Alkaloids did not appear in any fraction, suggesting that this compound might be too low to be detected by the TLC spray

reagent to be observed with the eyes. Since alkaloids were found in the extract, they must have been found in any fractions. The sensitivity is the limitation of TLC spray reagents (Zhang et al., 2021). It might be found in the fractions if we used the colorimetric method using a spectrophotometer since instrumental analysis would be more precise. The instrumental analysis will provide more sensitivity, resulting in more reliable data (Zhang et al., 2021). Alkaloids in *T. diversifolia* leaves seem to be the lowest compounds found compared to others, e.g., terpenoids, polyphenols, and flavonoids. Studies showed that only two alkaloids are found in *T. diversifolia* leaves (Zhao et al., 2012).

Table 2. Anti-inflammatory activity of *T. diversifolia* leaves ethanolic extract and its fractions. *Description*: *IC_{50} data was shown as mean ± SD (n=3); different letter notations in the same column indicate significant differences (LSD, $p<0.01$) in red Blood Cells (RBC).

Sample	IC_{50} (µg/mL)*	
	RBC membrane stabilization	**Protein denaturation inhibition**
Sodium diclofenac	60.315±0.729[a]	2.171±0.025[f]
Ethanolic extract of *T. diversifolia* leaves	78.427±0.594[b]	2.529±0.044[g]
n-hexane fraction of *T. diversifolia* leaves	94.500±0.206[c]	3.745±0.085[h]
Dichloromethane fraction of *T. diversifolia* leaves	89.268±0.224[d]	2.937±0.036[i]
Methanol-water fraction of *T. diversifolia* leaves	92.236±0.570[e]	3.502±0.021[j]

The ethanolic extract of *T. diversifolia* leaves and its fractions were then evaluated for their phenolic and flavonoid content. The total phenolic and flavonoid content is shown in Table 5. All the phenolic and flavonoid content were significantly different among the samples. The ethanolic extract of *T. diversifolia* leaves contained the highest phenolic compound. The ethanolic extract is more complex than the fractions, suggesting that the compounds in the ethanolic extract were being divided further into fractions (López-Cabeza et al., 2024). Thus, the ethanolic extract contained the highest phenolic compounds compared to its fractions.

Table 3. Antioxidant properties of *T. diversifolia* leaves ethanolic extract and its fractions. Description: *IC_{50} data was shown as mean ± SD (n=3), Different letter notations indicate significant differences (LSD, $p<0.01$), **DPPH antioxidant activity classification based on Phongpaichit et al. (2007).

Sample	IC_{50} ((µg/mL)*	Antioxidant category**
Vitamin C	2.534 ± 0.118	Very strongly active
Ethanolic extract of *T. diversifolia* leaves	22.592 ± 0.468[a]	Strongly active
n-hexane fraction of *T. diversifolia* leaves	67.755 ± 0.674[b]	Moderately active
Dichloromethane fraction of *T. diversifolia* leaves	51.105 ± 0.805[c]	Moderately active
Methanol-water fraction of *T. diversifolia* leaves	27.218 ± 0.848[d]	Strongly active

On the other hand, the highest flavonoid content was measured in the n-hexane fraction of *T. diversifolia* leaves. Some flavonoid compounds are non-polar polymethoxy aglycones or isoflavones, in which sugar groups or glycoside forms are released (Satria et al., 2022). However, there is a possibility that the total flavonoid content obtained does not show the actual

results. It is because extracts and fractions already have a color before being reacted; for example, the n-hexane fraction is green because it contains a lot of chlorophyll. Chlorophyll will be detected at a wavelength of 320-430 nm (Christiana et al., 2008), while the wavelength used for flavonoid detection with $AlCl_3$ is at a wavelength of 425 nm. At the test wavelength, chlorophyll is absorbed, so the calculated flavonoid content is above the actual value.

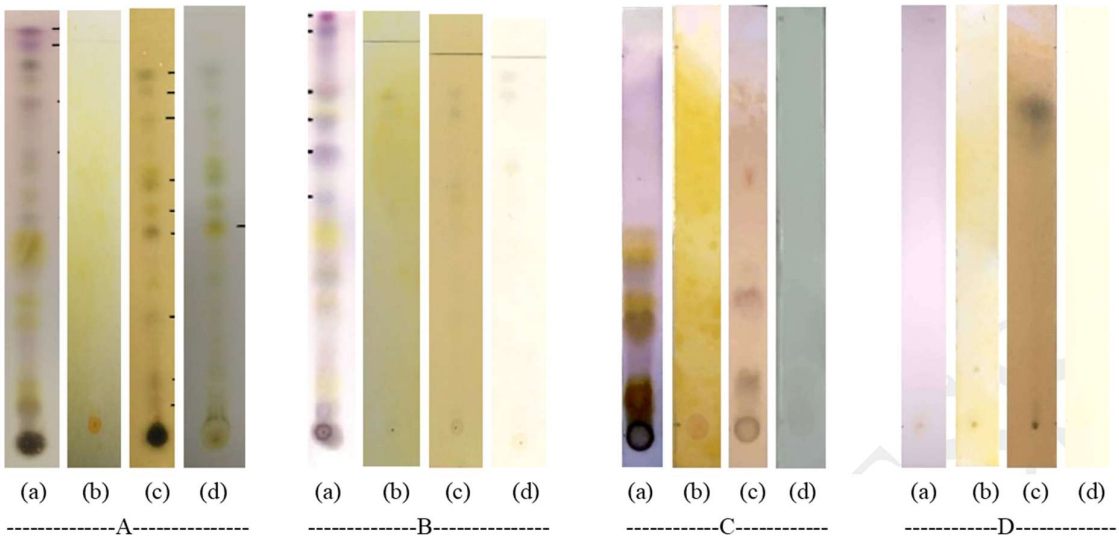

Figure 1. Chromatogram of phytochemical screening on ethanolic extract (A), n-hexane fraction (B), dichloromethane fraction (C), and methanol-water fraction of *T. diversifolia* leaves. The TLC was done using silica gel 60 F_{254} as the stationary phase. The mobile phase used was n-hexane (Merck): ethyl acetate (Merck) (3:2) for ethanol extract (A); toluene (Merck): ethyl acetate (Merck) (7:3) for n-hexane fraction (B); n-hexane (Merck): ethyl acetate (Merck) (2:1) for dichloromethane fraction (C); and n-hexane (Merck): ethyl acetate (Merck): methanol (Merck) (1:1:2) for methanol-water fraction (D). The chromatogram sprayed using respective spray reagents for specific compounds, i.e., anisaldehyde sulfuric acid for terpenoids, characterized by purplish spots (a); Dragendorff's reagent for alkaloids, characterized by orangish spots (b); $FeCl_3$ for polyphenols, characterized by blackish spots (c); and boric citrate for flavonoids, characterized by yellowish spots which disappeared quickly (d). Smear color will lead to undetectable compounds visually (Zhang et al., 2021).

Some fractions, i.e., n-hexane and dichloromethane fractions of *T. diversifolia* leaves, resulted in higher flavonoid content than phenolic content. Theoretically, flavonoids are part of phenolic compounds. It is unlikely to have a higher flavonoid quantification than phenolic compounds. However, sometimes, the researchers found the same anomaly, the flavonoid content they had was higher than the phenolic compound (Yu et al., 2021; Zulkifli et al., 2020). Nevertheless, there needs to be a brief explanation of this phenomenon. Not all phenolic compounds could be determined by the Folin Ciocalteu reagent or simply because the reference substances used as standard are different.

The multiple regression analysis showed that total phenolic and flavonoid content contributed 92.1% and 90.6% of anti-inflammatory and antioxidant activity, respectively (Figure 2). The unstandardized predicted value was for anti-inflammatory (both from RBC

membrane stabilization and protein denaturation inhibition method) and antioxidant activity. Total phenolic and flavonoid content significantly affected anti-inflammatory and antioxidant activity and were classified as highly correlated (Eryani et al., 2022).

Table 4. Phytochemical screening of *T. diversifolia* leaves ethanolic extract and its fractions.

Sample	Terpenoids	Alkaloids	Polyphenols	Flavonoids
Ethanolic extract of *T. diversifolia* leaves	+	+	+	+
N-hexane fraction of *T. diversifolia* leaves	+	-	+	+
Dichloromethane fraction of *T. diversifolia* leaves	+	-	+	+
Methanol-water fraction of *T. diversifolia* leaves	-	-	+	+

Based on Pearson correlation analysis (Table 6), the correlation of total phenolic content to the anti-inflammatory activity (both from RBC membrane stabilization and protein denaturation inhibition method) was negative, meaning that the higher the phenolic content, the lower the IC_{50} on RBC membrane stabilization and protein denaturation inhibition. The total phenolic content played a significant correlation to RBC membrane stabilization with the value of -0.768 (high negative correlation, $p<0.01$), but no significant correlation to protein denaturation inhibition and antioxidant activity with a correlation coefficient of -0.626 (moderate negative correlation) and -0.403 (low negative correlation), respectively. The total flavonoid content negatively correlated to antioxidant activity, suggesting that the higher the total flavonoid content, the lower the IC_{50} to antioxidant activity.

Table 5. Total phenolic and flavonoid content of *T. diversifolia* leaves ethanolic extract and its fractions. *Description:* *Data was shown as mean ± SD (n=3); different letter notations in the same parameter indicate significant differences (LSD, $p<0.01$). GAE = gallic acid equivalent. QE = quercetin equivalent.

Sample	Total phenolic content* (mg GAE/g sample)	Total flavonoids content* (mg QE/g sample)
Ethanolic extract of *T. diversifolia* leaves	32.447 ± 0.337^{a}	21.374 ± 0.585^{e}
n-hexane fraction of *T. diversifolia* leaves	12.035 ± 0.262^{b}	135.103 ± 0.517^{f}
Dichloromethane fraction of *T. diversifolia* leaves	16.287 ± 0.730^{c}	63.661 ± 1.056^{g}
Methanol-water fraction of *T. diversifolia* leaves	26.349 ± 0.330^{d}	11.901 ± 0.119^{h}

On the other hand, the correlation between total flavonoid content and anti-inflammatory activity (RBC membrane stabilization and protein denaturation inhibition method) was positive. This anomaly is related to the anomaly in the total flavonoid content calculation due to the previously described challenges. There was no significant correlation between total flavonoid content and anti-inflammatory and antioxidant activity. The correlation coefficient on total flavonoid content to RBC membrane stabilization was 0.433 (low positive correlation), protein denaturation inhibition was 0.47 (low positive correlation), and antioxidant was -0.233 (negligible negative correlation) (Mukaka, 2012).

These findings would complete the anti-inflammatory mechanism of action of ethanolic extract of *T. diversifolia* leaves and its fraction, following red blood cell membrane stabilization and protein denaturation inhibition. The antioxidant properties likely contributed more to RBC

membrane stabilization than protein denaturation inhibition. The phenolic and flavonoid content contributed most to antioxidant and RBC membrane stabilization rather than protein denaturation inhibition (Figure 3).

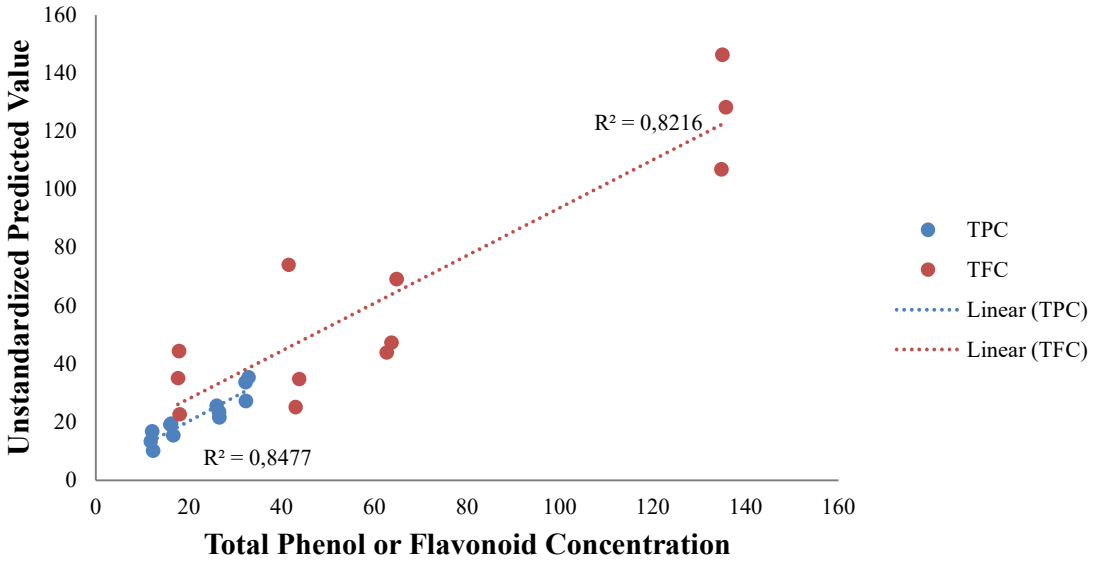

Figure 2. Multiple regression analysis showed that total phenolic (TPC) and flavonoid content (TFC) contributed significantly to the anti-inflammatory and antioxidant activity with high correlation.

The ethanolic extract of *T. diversifolia* leaves showed the highest anti-inflammatory and antioxidant activity. It contained terpenoids, alkaloids, polyphenols, and flavonoids. Terpenoids inhibit cyclooxygenase and suppress c-Jun N-terminal kinase, leading to the inhibition of pro-inflammatory mediators (Subedi & Yumnam, 2021). Alkaloids might inhibit either cyclooxygenase 1 or 2, as well as lypooxygenase, resulting in inhibition of pro-inflammatory mediators (Souto et al., 2011). Polyphenols stabilize membrane structure and function by forming stronger hydrogen bonds (Bouhlali et al., 2020).

Table 6. Pearson correlation analysis on total phenolic and flavonoid content to anti-inflammatory and antioxidant activity. *Description*: * correlation is significant at a 99% confidence level ($p<0.01$).

Parameter	Pearson correlation value	
	Total phenolic content	Total flavonoid content
RBC stabilization membrane	-0.626	0.470
Protein denaturation inhibition	-0.768*	0.433
Antioxidant	-0.403	-0.801

Mechanism of actions of flavonoids as anti-inflammatory and antioxidant agents are mediated by inhibition of regulatory proteins, e.g., protein kinases and phosphodiesterases, resulting in decreased signal transduction; scavenger activity on free radicals, resulting in decreased oxidative stress; inhibition of phospholipase A2, cyclooxygenase, and lipoxygenase,

causing decrease release of pro-inflammatory mediators; modulation of transcription factors: NF-κB, GATA-3, STAT-6, resulting in decreased transcription of pro-inflammatory genes; and also inhibition of cell activation, maturation, signaling transduction, secretory processes of immune cells causing immature immune cells, decreased cell proliferation, and decreased release of pro-inflammatory cytokines (Maleki et al., 2019). The possible mechanism of actions of compounds contained in ethanolic extract of *T. diversifolia* leaves are shown in Figure 3. The fact that the ethanolic extract of *T. diversifolia* leaves had the highest anti-inflammatory and antioxidant activity compared to that of the fractions suggests that these compounds in the extract might exhibit synergistic additive effects, not antagonistic ones—the total phenolic and flavonoid concentrations in a plant correlate with the anti-inflammatory and antioxidant activity. The higher polyphenols lead to higher anti-inflammatory and antioxidant activity (Benjamin et al., 2024; Rini et al., 2023). Nevertheless, further research needs to be done to confirm these possibilities.

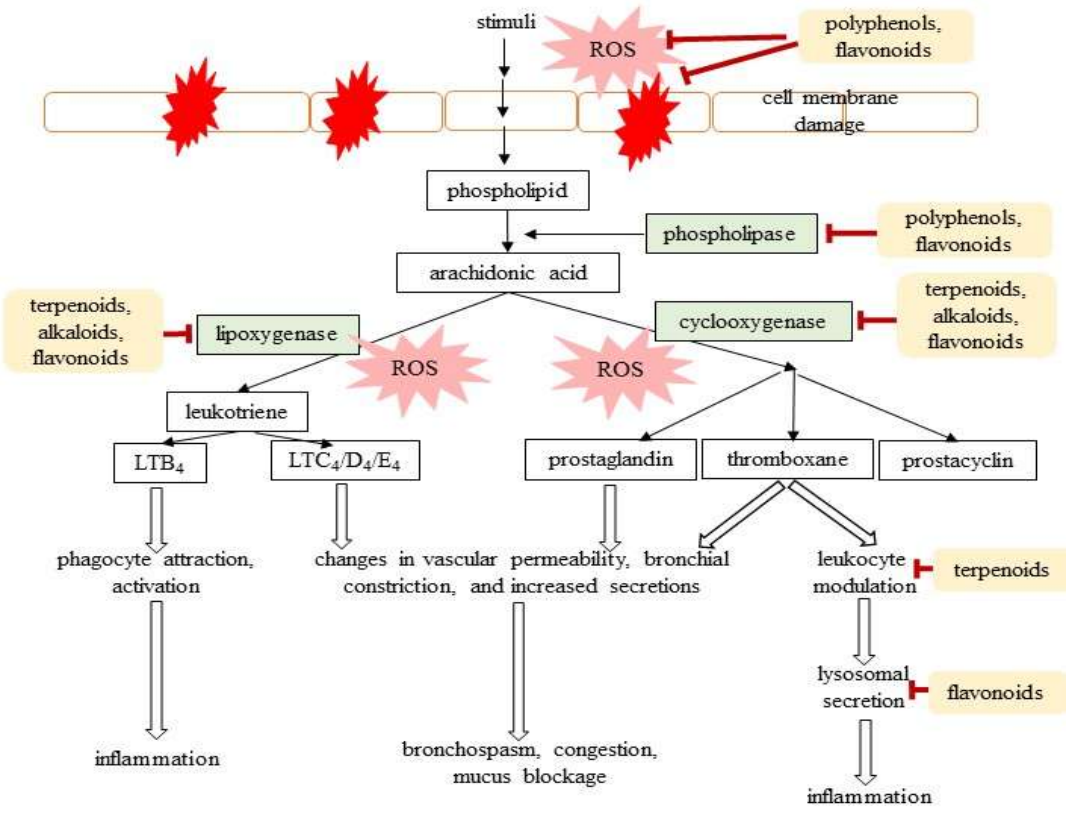

Figure 3. A proposed mechanism on how the compounds contained in ethanolic extract of *T. diversifolia* leaves ethanolic extract and its fractions act as anti-inflammatory and antioxidant (Bouhlali et al., 2020; Katzung et al., 2018; Maleki et al., 2019; Souto et al., 2011; Subedi & Yumnam, 2021). *Description*: Reactive Oxygen Species (ROS), ⊣ (inhibit/block).

The ability to block or inhibit inflammation and the antioxidants featured by ethanolic extract of *T. diversifolia* leaves will act as a possible mechanism of the antiproliferative and anticancer activities of *T. diversifolia* leaves. Inflammation is one of the hallmarks of cancer

(Hanahan, 2022). Thus, anti-inflammatory drugs have been repurposed for anticancer therapy (Ozleyen et al., 2023). On the other hand, the antioxidant properties of natural products will scavenge the free radicals in our body and control inflammatory responses, supporting the already considered anti-inflammatory activity. These two mechanisms of action, antioxidant and anti-inflammatory activities, may work synergistically in cancer chemoprevention (Jongrungraungchok et al., 2023).

4. CONCLUSION

Based on the results, we can conclude that the ethanolic extract of *T. diversifolia* leaves had the best anti-inflammatory properties compared to its fractions based on both methods and antioxidant activity. The antioxidant activity contributed more to RBC membrane stabilization compared to protein denaturation inhibition. The phenolic and flavonoid content contributed more to antioxidant and red blood cell membrane stabilization. The ethanolic extract of *T. diversifolia* leaves contained terpenoids, alkaloids, polyphenols, and flavonoids that might exert synergistic or additive effects on anti-inflammatory and antioxidant properties. Nevertheless, research is still needed to confirm these possibilities.

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CONFLICT OF INTEREST

All authors declared that there was no conflict of interest.

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