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Propolis is a traditional food with significant health benefits owing to its rich polyphenol content. An extraction process is essential to obtain bioactive ingredients, including flavonoids and phenolic acids. This study aimed to optimise the extraction of polyphenol-rich compounds from poplar-type propolis and evaluate their anti-inflammatory effect and underlying mechanisms in lipopolysaccharide (LPS)-induced inflammation. By employing two Box-Behnken design experiments, four key variables – extraction time, temperature, ethanol concentration, and solid-to-liquid ratio – were systematically optimised to maximise the yields of phenolic acids and flavonoids. Subsequently, a two-step extraction method was developed to maximise the recovery of phenolic acids and flavonoids via response surface optimisation. The anti-inflammatory effect of the propolis extracts was evaluated in LPS-treated bovine mammary epithelial cells and in a mouse mastitis model. The results showed that a high concentration of ethanol (85%) was optimal for extracting non-polar polyphenols, such as flavonoids, whereas a low concentration of ethanol (23%) was more effective for extracting polar polyphenols, such as phenolic acids. The two-step method combined these ethanol extracts to maximise polyphenol yield. The optimal extraction conditions were 23/25 h extraction time, 46/41 °C temperature, and 1:14/1:11 (g/mL) solid-to-liquid ratio for 85% and 23% ethanol, respectively. This approach yielded a total flavanol content of 531.6 mg rutin/g propolis extract and a total phenolic content of 214.3 mg gallic acid/g propolis extract. The anti-inflammatory potential of the combined extracts was assessed in the context of mastitis, a condition characterised by inflammation and compromised milk quality. In vitro, the propolis extract significantly increased bovine mammary epithelial cell viability and downregulated the mRNA levels of pro-inflammatory cytokines including TNF- α , IL-1 β , and IL-6. In vivo, the propolis extract effectively alleviated histopathological lesions and suppressed the production of inflammatory cytokines. Furthermore, the combined extract preserved the integrity of blood-milk barrier by upregulating tight junction proteins. RNA-seq analysis revealed that the primary genes involved were enriched in the PI3K/AKT signalling pathway. In summary, optimised extraction methods can be readily translated into more efficient production processes for high-quality propolis. The anti-inflammatory properties of propolis extracts enhance their potential therapeutic applications in the treatment of inflammatory diseases.

Anti-inflammatory Activity of Polyphenol-Rich Compounds from Poplar-Type Propolis Using an Optimized Extraction Method

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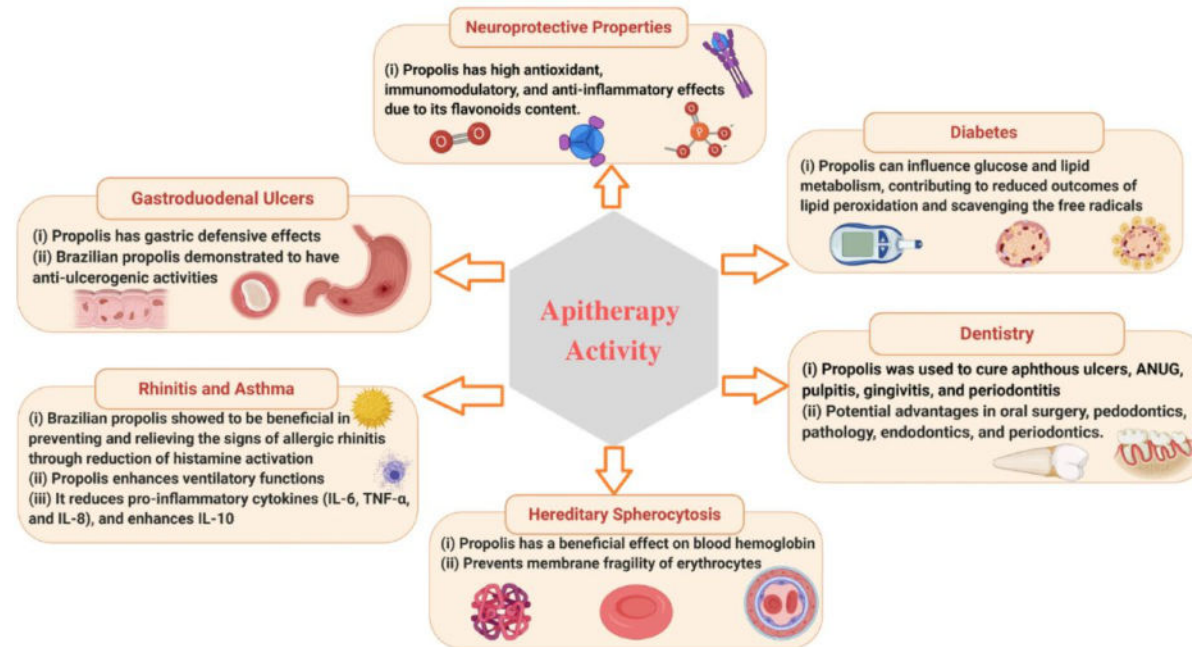
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Propolis is a resinous material that honeybees (*Apis mellifera*) collect from various species of plant resins to protect their hives from invaders and seal cracks.



Chemo-geographical variation

Poplar-type

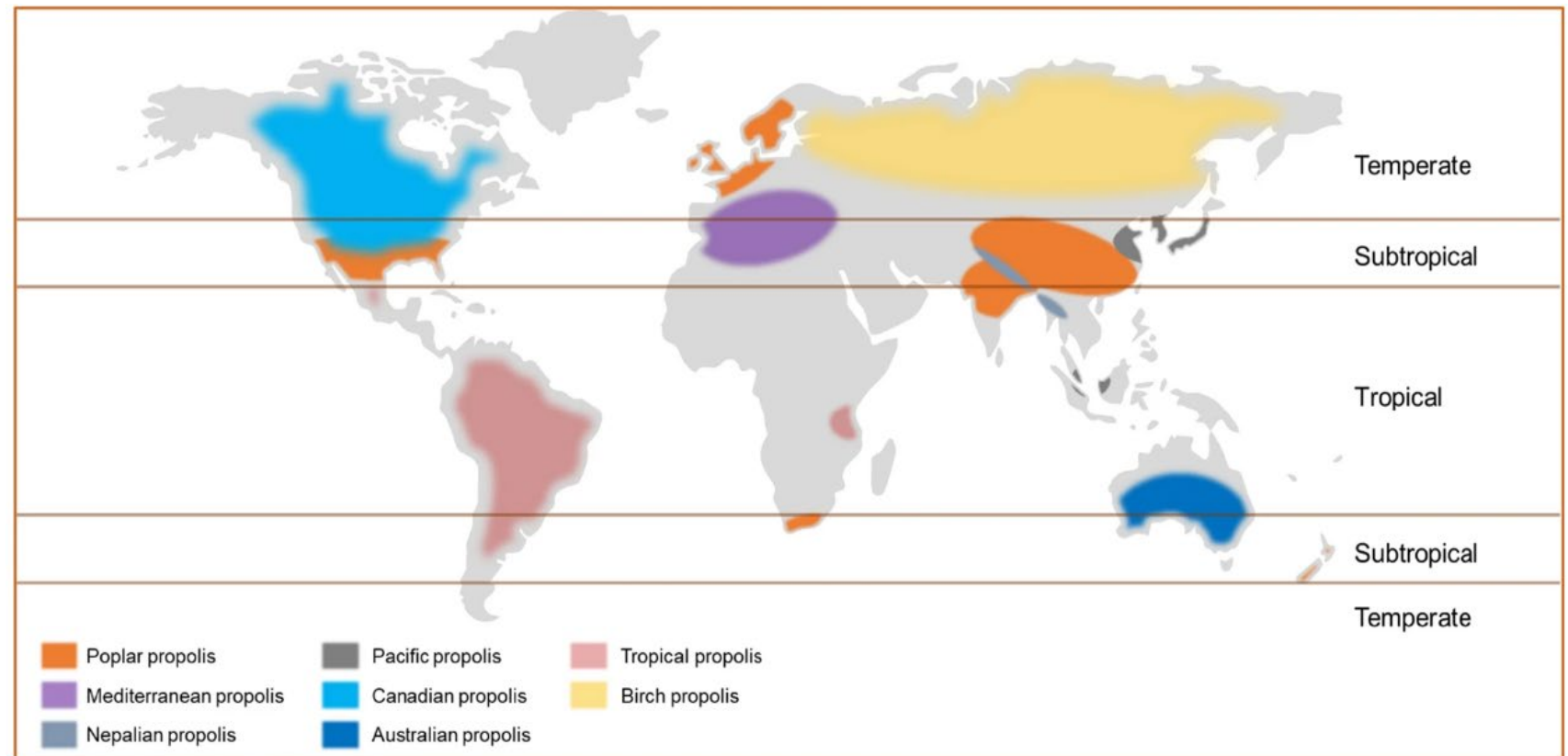
Baccharis-type

Birch-type

Macaranga-type

Dalbergia-type

...



Chemo-geographical variation

Poplar-type

Baccharis-type

Birch-type

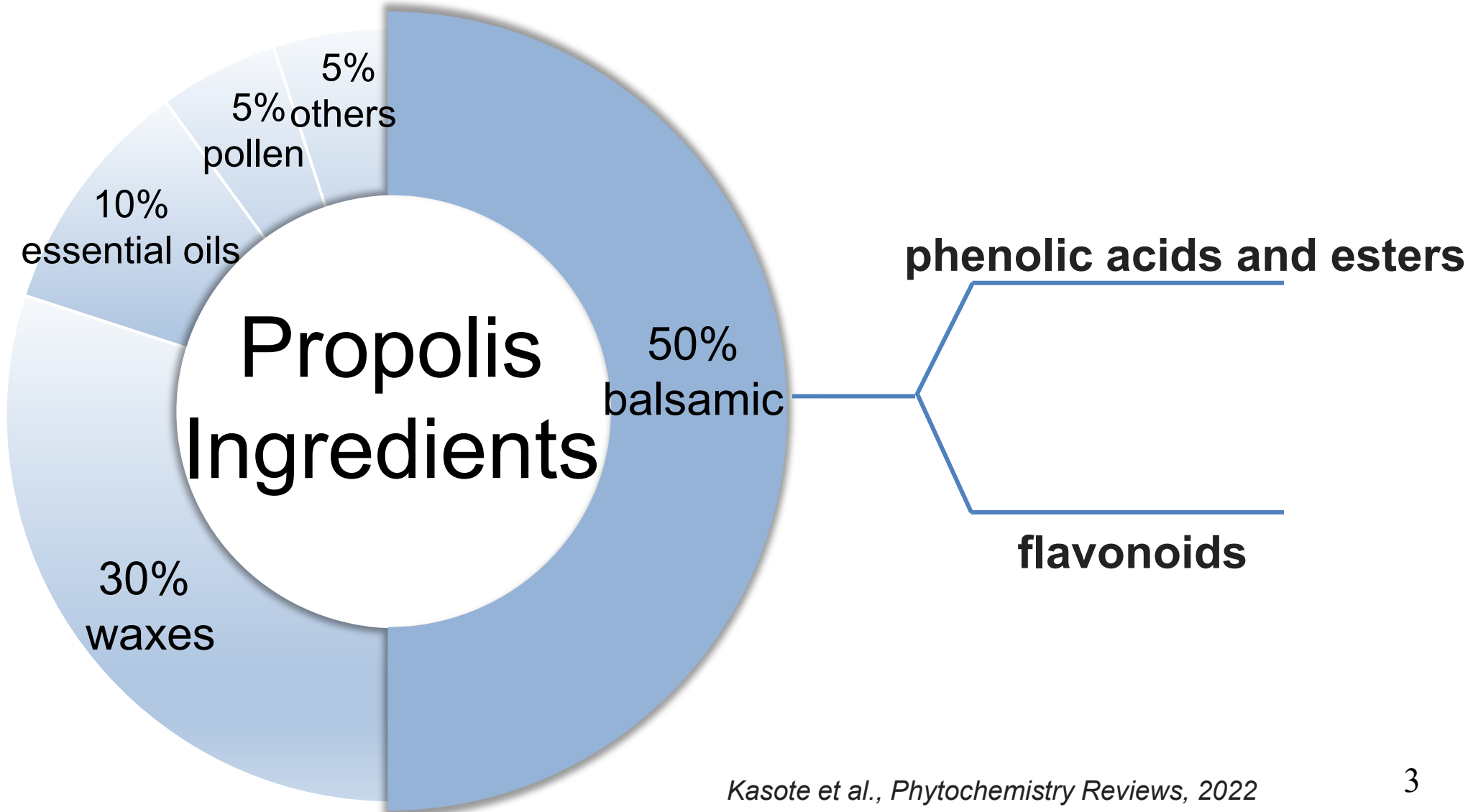
Macaranga-type

Dalbergia-type

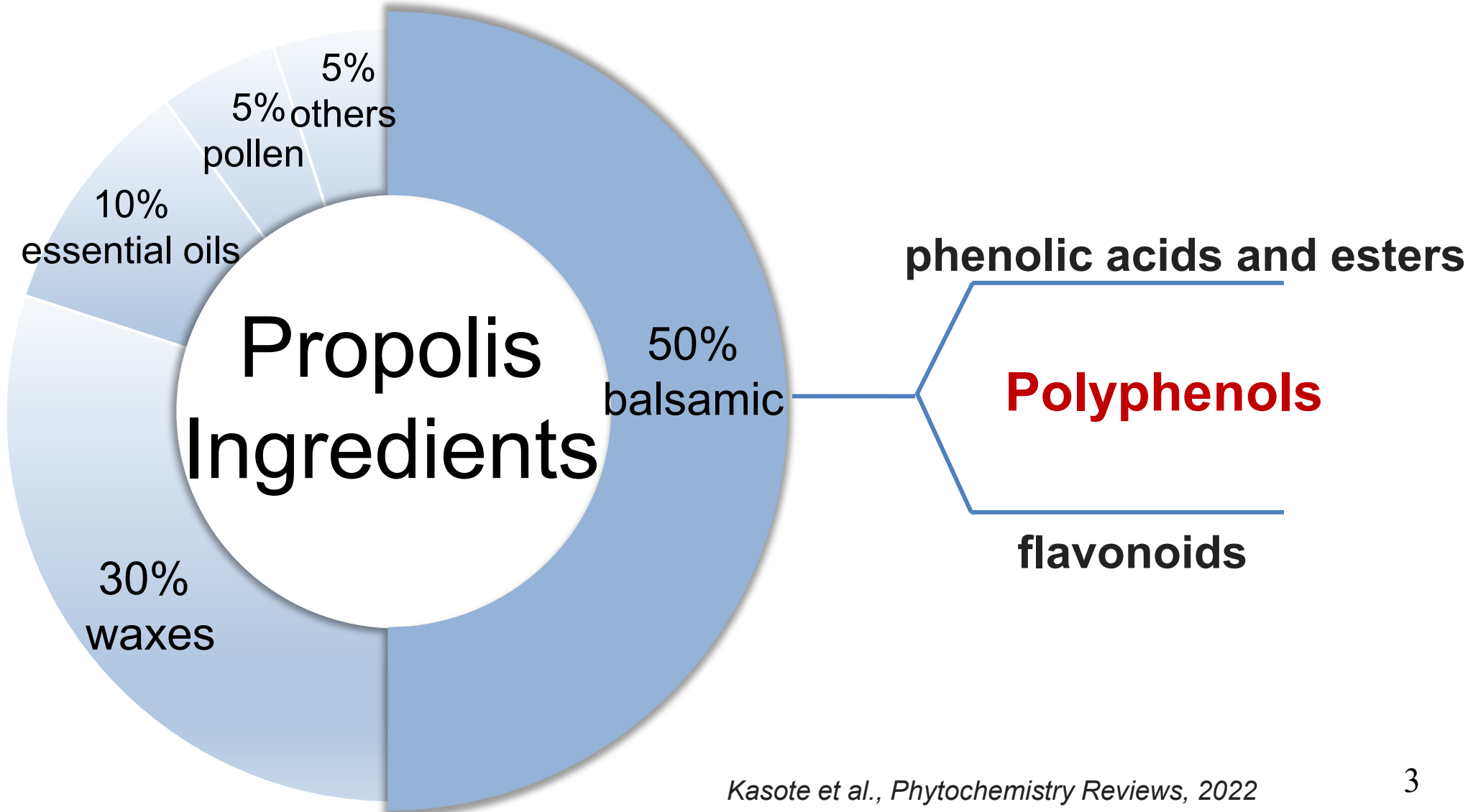
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Phytochemical composition



Phytochemical composition



Influencing factors_Maceration

Solvent, temperature, frequency, time, solid-liquid ratio

Geographic origin	Ethanol concentration	References
Morocco	70%	Touzani et al. (2019)
Turkey	70%	Ozdal et al. (2019)
Poland	70%	Pobiega et al. (2019)
Brazil	80%	de Oliveira Reis et al. (2019)
Tunisia	80%	Gargouri et al. (2019)
Greece	80%	Lagouri et al. (2014)
China	95%	Wang et al. (2016)

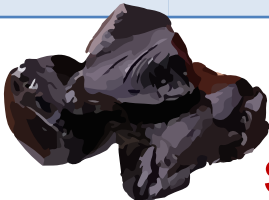
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liquid



solid

- **Which is the optimal approach?**

95% ethanol?

70% ethanol ?

80% ethanol ?

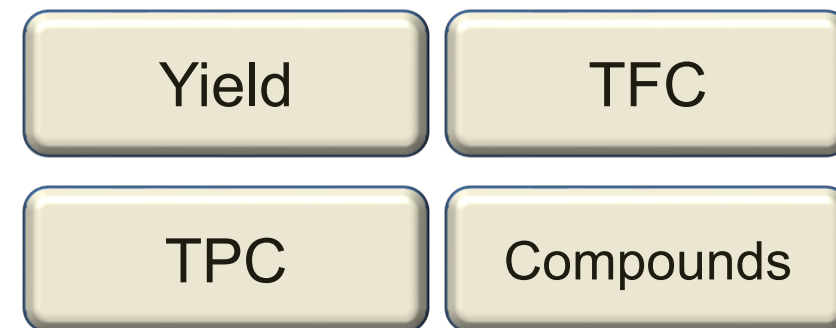
- **Defined by what metrics?**

Total flavonoids?

Total phenolic?



Factors



Evaluation

Single-factor
experiments

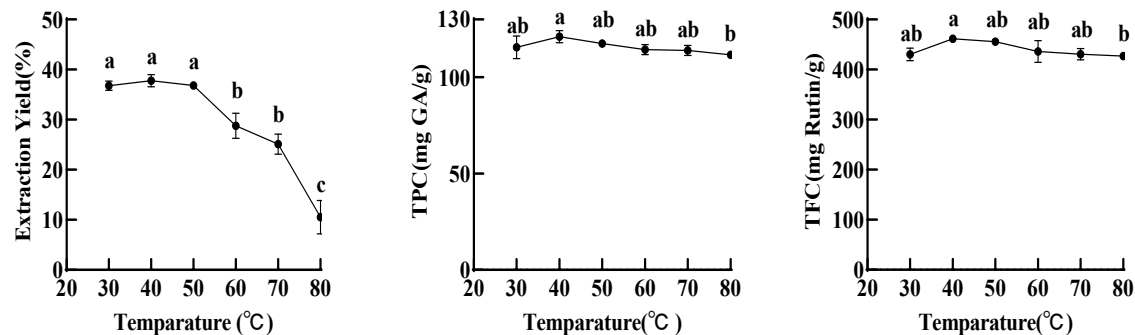
Response surface
methodology

Characterization

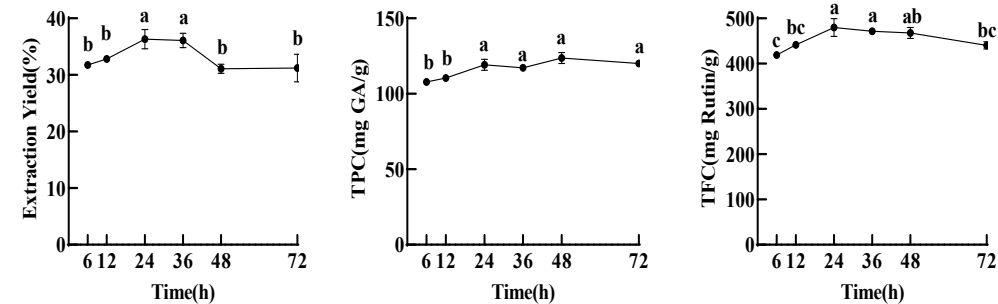
Single-factor experimental design

Single factor	Level
Temperature/°C	30, 40, 50, 60, 70, 80
Time/h	6, 12, 24, 36, 48, 72
Solid-Liquid Ratio/g/mL	1 : 4, 1 : 6, 1 : 8, 1 : 10, 1 : 20, 1 : 30, 1 : 40, 1 : 50
Ethanol Concentration/%	0, 20, 40, 60, 70, 75, 80, 85, 90, 95, 100

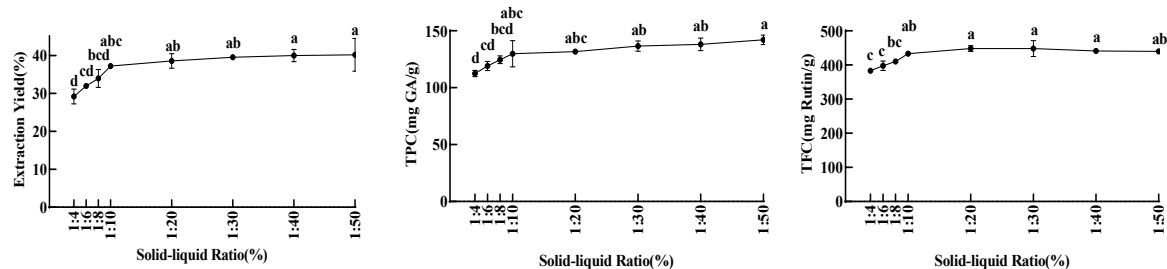
Single-factor experiment



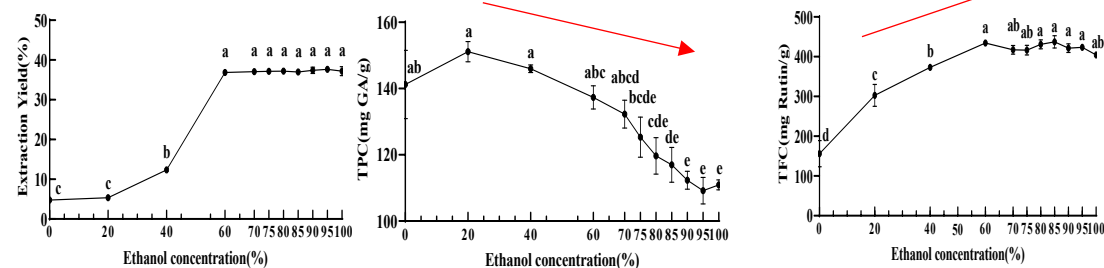
Temperature_40°C



Time_24h



Solid-Liquid ratio_1:10



Ethanol concentration

Response surface methodology

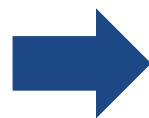
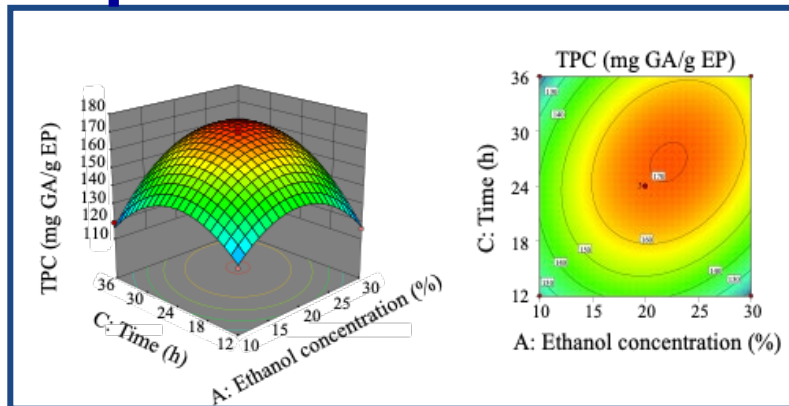
Box-Behnken design factor level for **TPC**

Levels	Factor			
	A (%)	B (°C)	C (h)	D (g/mL)
-1	10	30	12	1:5
0	20	40	24	1:10
1	30	50	36	1:15

Box-Behnken design factor level for **TFC**

Levels	Factor			
	A (%)	B (°C)	C (h)	D (g/mL)
-1	60	30	12	1:5
0	80	40	24	1:10
1	100	50	36	1:15

Optimization results



● TPC

Ethanol
concentration/%
23

Solid-Liquid
ratio

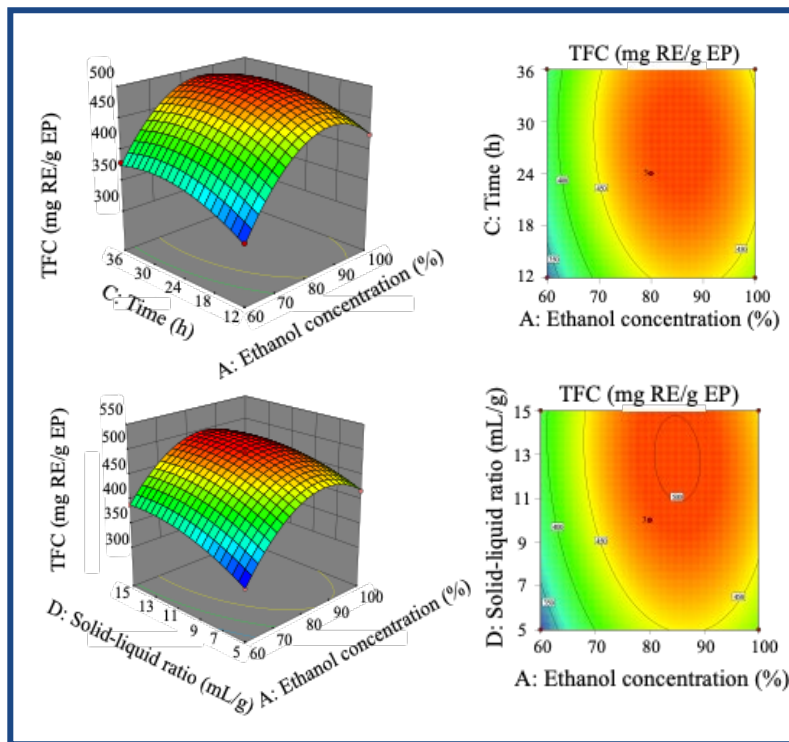
1: 11

Time/h

25

Temperature/°C

41



● TFC

Ethanol
concentration/%
85

Solid-Liquid
ratio

1: 14

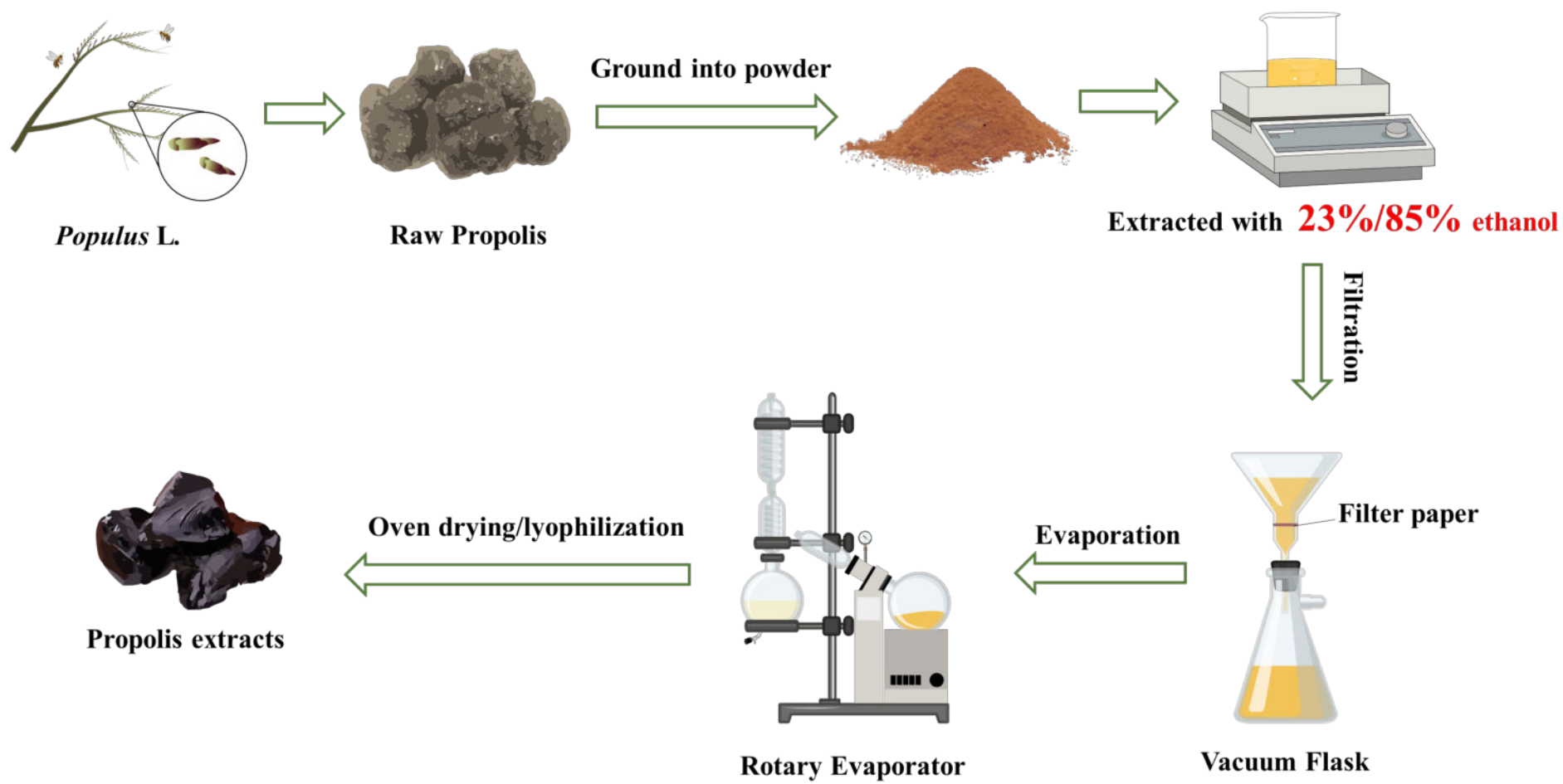
Time/h

23

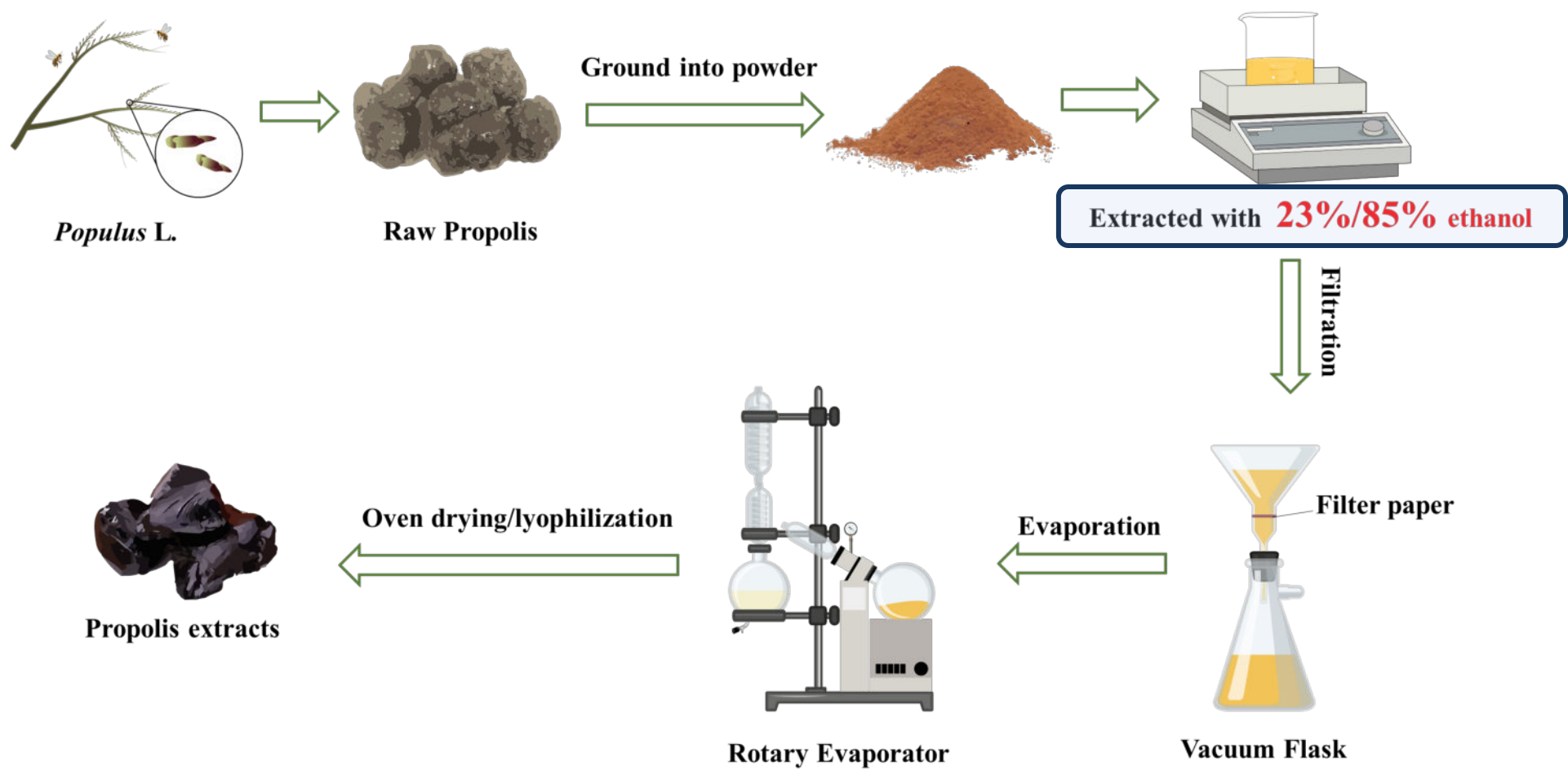
Temperature/°C

46

Construction of two-step extraction



Construction of two-step extraction



Construction of two-step extraction

➤ 23% ethanol extract (P)

TPC	TFC	Total
172.51±2.70 ^a	295.32±13.79 ^b	467.83±11.22 ^c

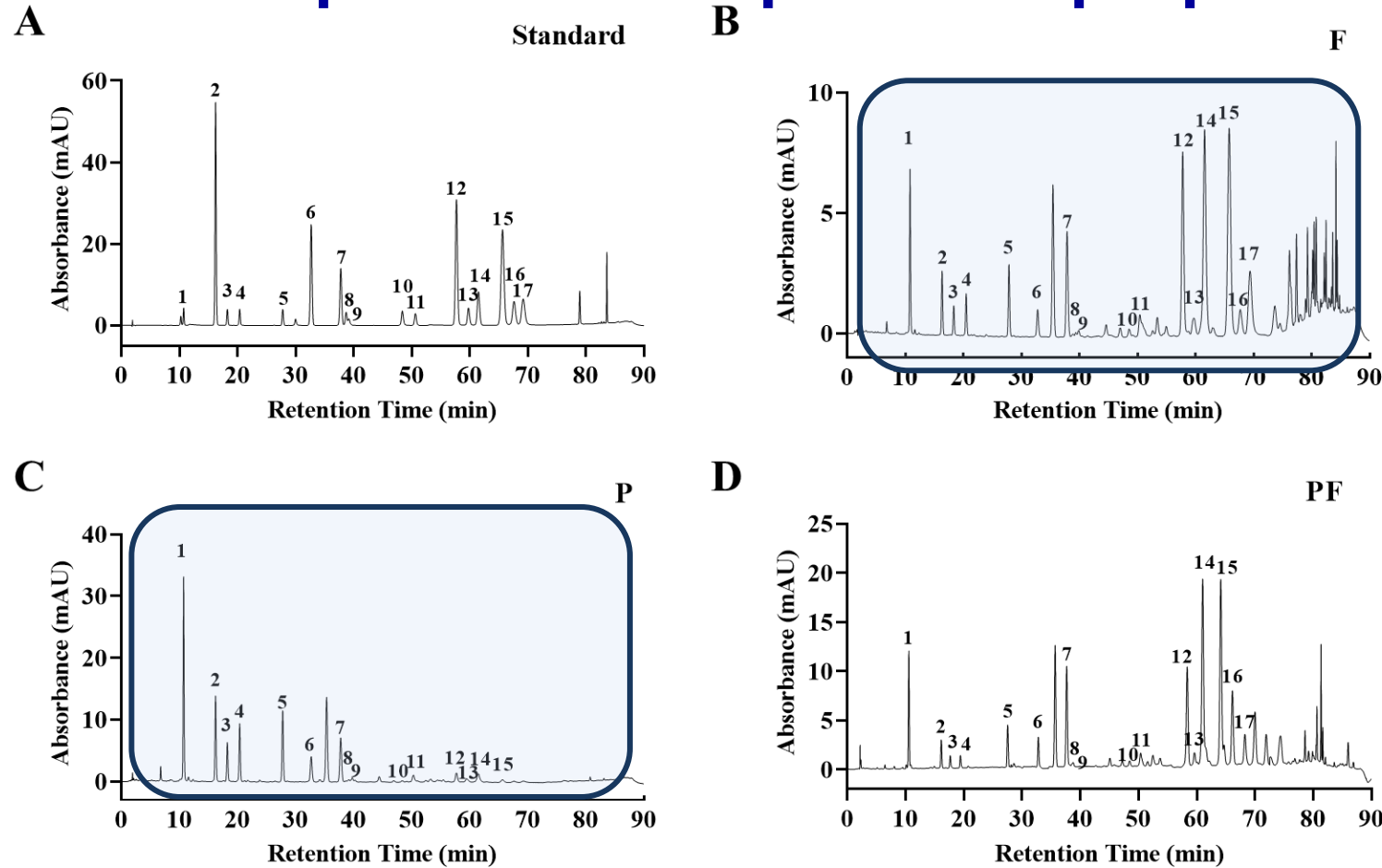
➤ 85% ethanol extract (F)

TPC	TFC	Total
111.45±3.40 ^c	493.50±1.83 ^a	607.95±5.06 ^b

➤ Two-step extracts
23% ethanol +85% ethanol (PF)

TPC	TFC	Total
157.21±1.92 ^b	471.13±13.41 ^b	628.34±11.86 ^a

HPLC of the 17 main phenolic compound in propolis extracts



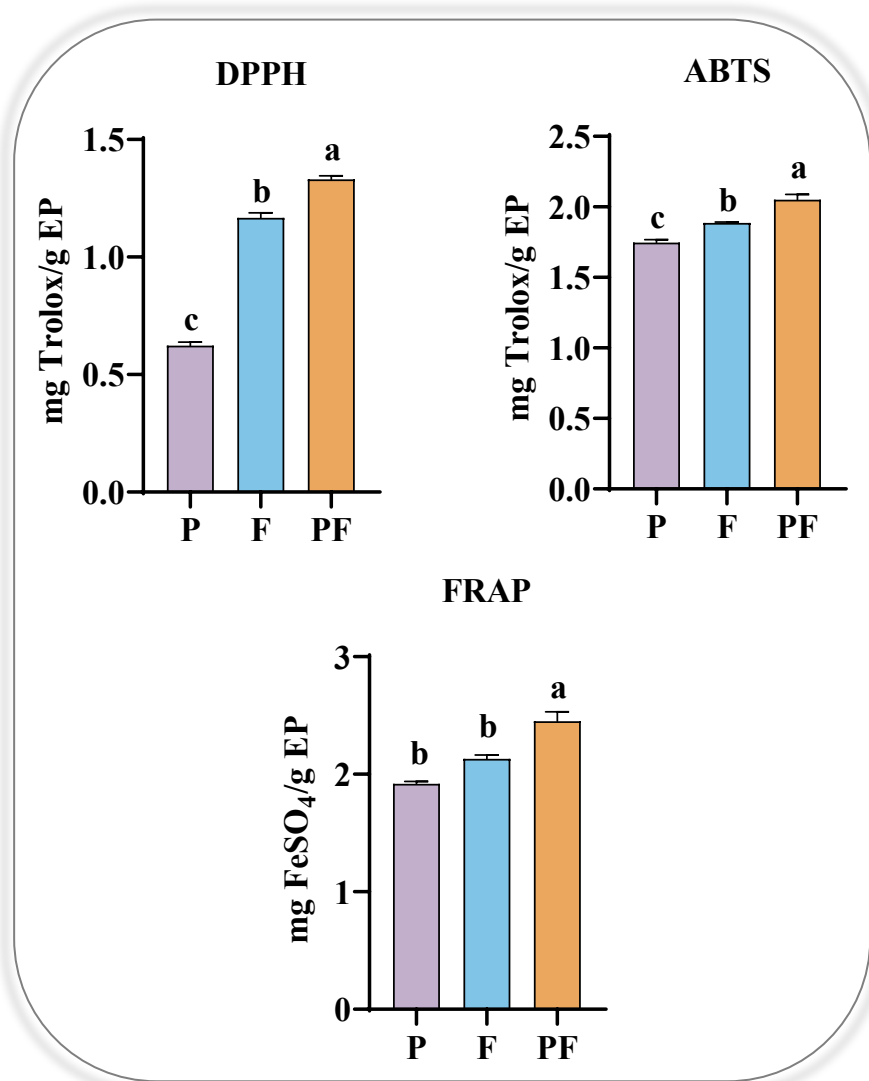
(A) Standard, (B) 85% ethanol extracts (F), (C) 23% ethanol extracts (P), (D) two-step extracts (PF).

1. Caffeic acid; 2. *p*-Coumaric acid; 3. Ferulic acid; 4. Isoferulic acid; 5. 3,4-Dimethoxycinnamic acid; 6. Cinnamic acid; 7. Pinobanksin; 8. Naringenin; 9. Quercetin; 10. Kaempferol; 11. Apigenin; 12. Pinocembrin; 13. Caffeic acid benzyl ester; 14. 3-O-acetylpinobanksin; 15. Chrysin; 16. Caffeic acid phenethyl ester; 17. Galangin.

HPLC of the 17 main phenolic compound in propolis extracts

No.	Compounds	Retention time (min)	Content (mg/g propolis extracts)		
			P	F	PF
1	caffeic acid	10.695	89.7±2.70 ^a	20.9±4.87 ^c	32.6±2.99 ^b
2	<i>p</i> -coumaric acid	16.207	20.1±0.54 ^a	3.99±0.73 ^b	3.84±0.06 ^b
3	ferulic acid	18.235	18.3±0.93 ^a	3.68±0.57 ^b	3.34±0.33 ^b
4	isoferulic acid	20.363	26.6±0.68 ^a	4.89±0.60 ^b	3.00±0.27 ^c
5	3,4-dimethoxycinnamic acid	27.776	31.1±1.36 ^a	8.69±0.61 ^b	8.80±0.34 ^b
6	cinnamic acid	32.675	3.16±0.18 ^a	0.93±0.05 ^c	1.47±0.02 ^b
7	pinobanksin	37.766	16.5±1.45	17.7±5.44	19.3±0.42
8	naringenin	38.696	0.16±0.11	0.35±0.36	0.16±0.11
9	quercetin	39.187	8.83±0.39 ^a	6.38±0.15 ^b	8.62±0.09 ^a
10	kaempferol	48.371	3.40±0.24	4.17±0.30	4.58±0.20
11	apigenin	50.621	7.80±1.17 ^a	4.48±0.80 ^b	6.99±0.53 ^a
12	pinocembrin	57.705	7.19±0.13 ^b	37.4±5.73 ^a	36.3±0.48 ^a
13	caffeic acid benzyl ester	59.774	7.23±0.25 ^b	11.7±1.56 ^a	8.21±0.91 ^b
14	3-O-acetylpinobanksin	61.49	12.2±1.39 ^c	78.3±6.22 ^b	95.1±9.58 ^a
15	chrysin	65.631	3.67±0.16 ^b	46.5±7.01 ^a	48.4±1.46 ^a
16	caffeic acid phenethyl ester	67.616	-	17.4±0.29 ^b	27.4±0.90 ^a
17	galangin	69.201	-	45.0±1.01 ^a	42.2±1.01 ^b
	Total		255.86±3.75 ^c	312.47±16.88 ^b	350.22±6.56 ^a

Antioxidant and antimicrobial activity *in vitro*

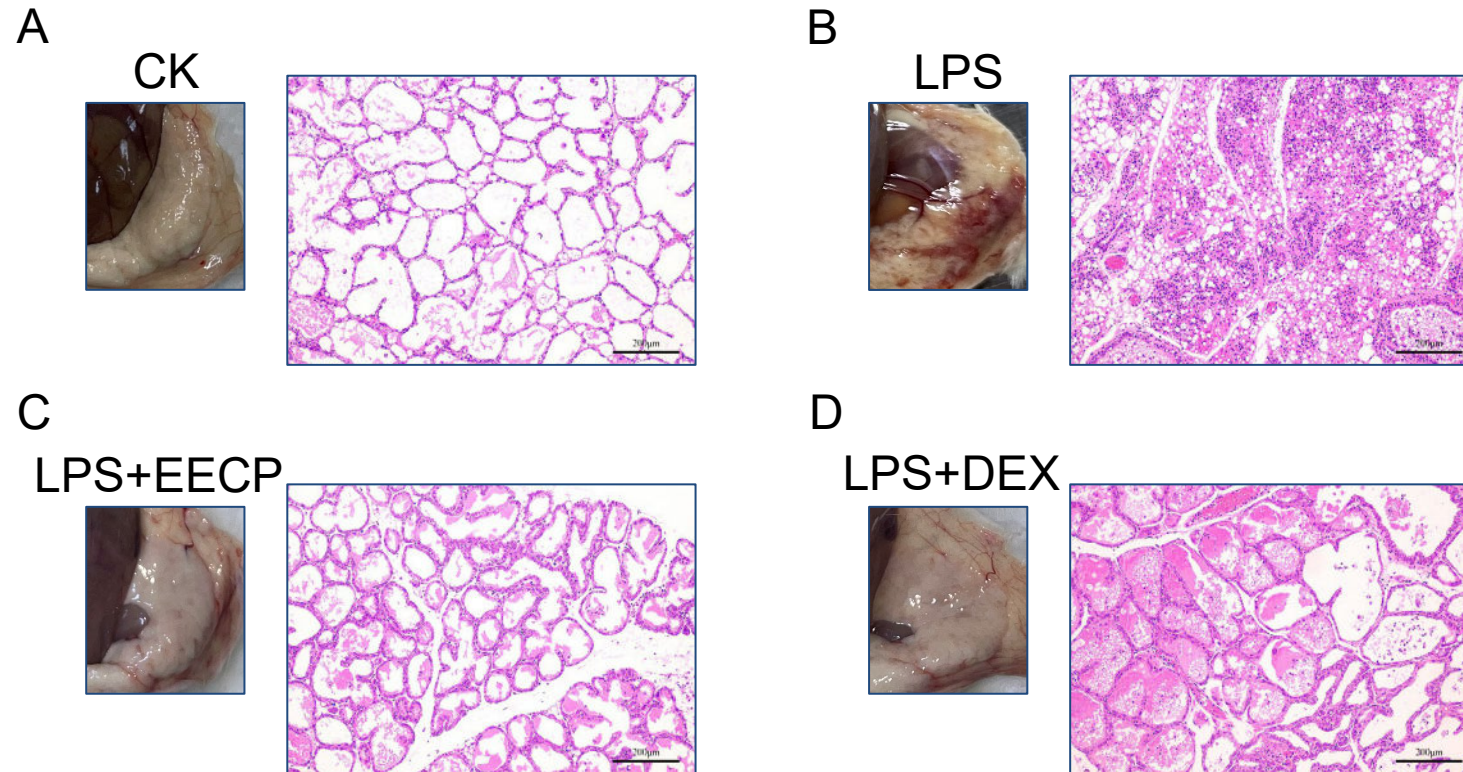


Sample	MIC (<i>E.coli</i>)	MIC (<i>S.aureus</i>)
	(ATCC 25922)/mg/ml	(ATCC 29213)/mg/ml
P	3.125	0.390625
F	1.5625	0.195313
PF	0.78125	0.097658

Antioxidant activity : PF > F > P

Antimicrobial activity : PF > F > P

Anti-Inflammatory activity *in vivo* (mouse mastitis model)

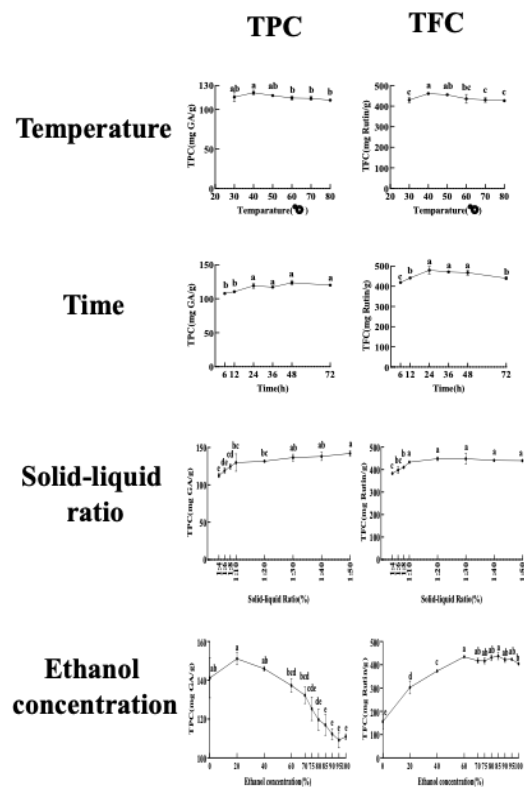


- LPS group exhibited hyperemia, edema, and significant neutrophil infiltration.
- Administration of **EECP** significantly **attenuated** these degenerative changes in the mammary gland tissues of mice.

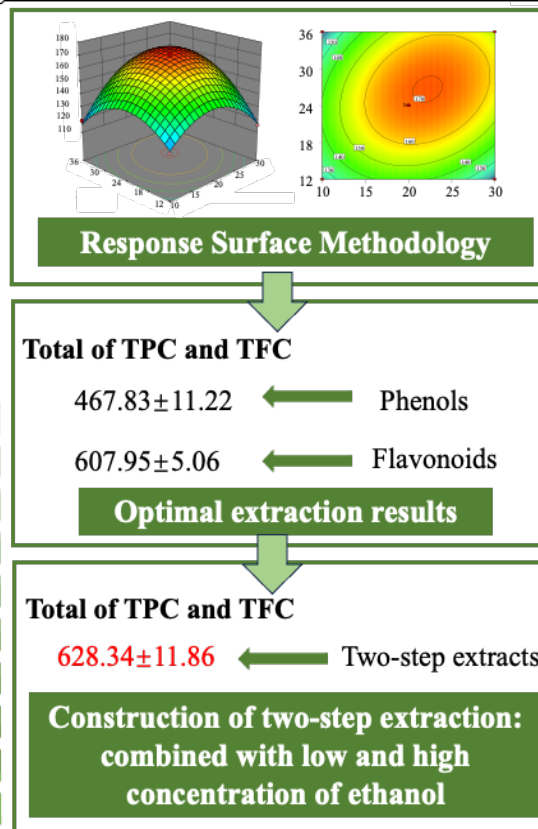
Maceration extraction



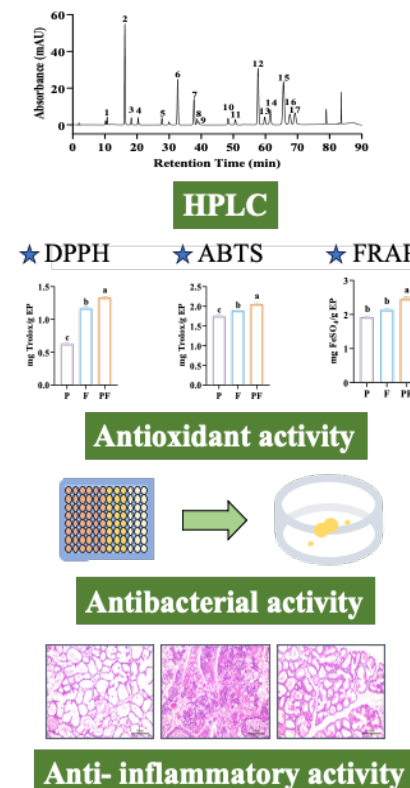
Single-factor analysis

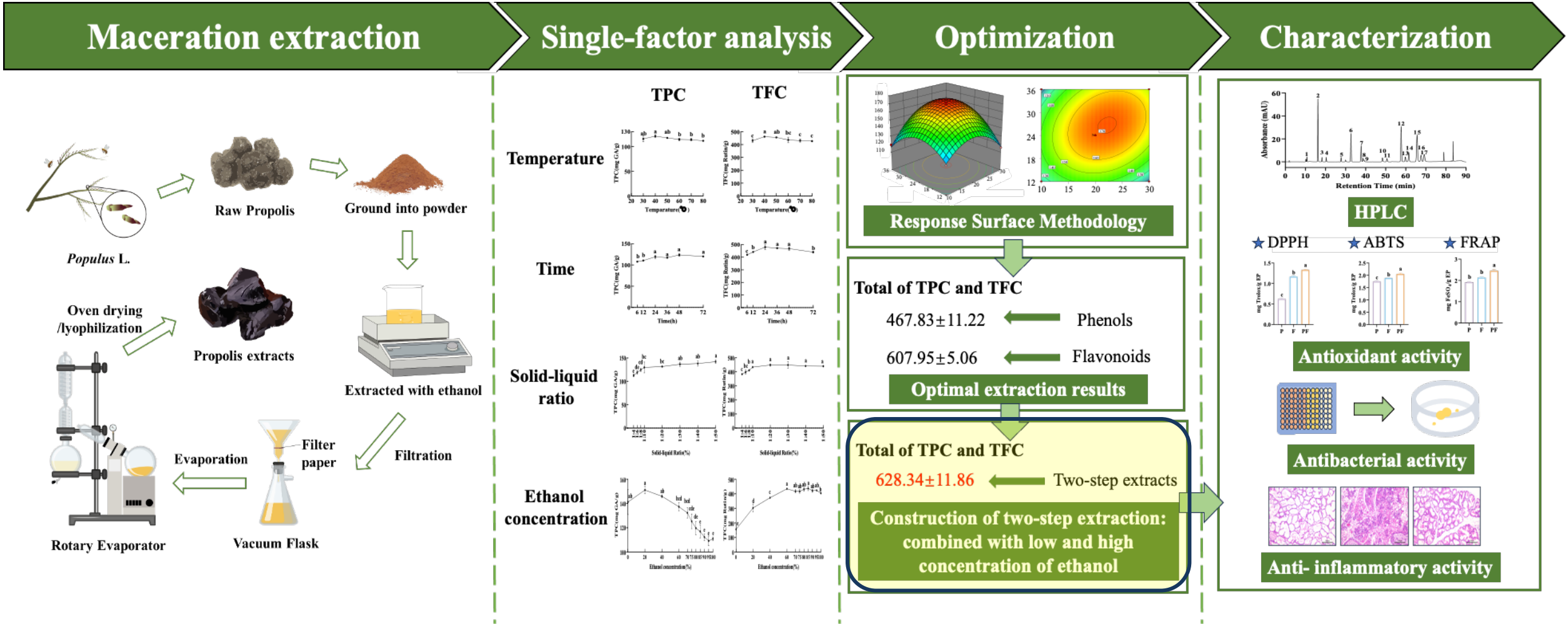


Optimization



Characterization





● Two-step extraction process allows for a more comprehensive recovery of the diverse polyphenol compounds in poplar-type propolis.



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Thank You

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