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Original Research Article

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Preliminary phytochemical profile, in vitro

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antioxidant and sun protective activities of *Alhagi*

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pseudalhagi and *Elaeagnus angustifolia* L.

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ABSTRACT

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Background and Objectives: *Alhagi pseudalhagi* and *Elaeagnus angustifolia* are among the medicinal plants used traditionally in Afghanistan. The aim of present work was to study *A. pseudalhagi* herbs and *E. angustifolia* leaves for their preliminary phytochemical profile, total phenolic content (TPC), total flavonoid content (TFC), *in vitro* antioxidant activity and sun protective factor (SPF).

Methods: Standard qualitative phytochemical tests were performed to detect various phytochemicals in the crude drugs. Prescribed methods were followed for determining TPC, TFC, and SPF of the plant extracts. To evaluate *in vitro* antioxidative potential, DPPH radical scavenging assay method was performed. All of the experiments were performed in three replications.

Results: Qualitative phytochemical tests revealed presence of carbohydrates, phenolics, flavonoids, sterols, tannins, and saponins in the plants extracts. The TPC of tested extracts ranged from 41.74 ± 1.22 to 203.76 ± 2.07 mg gallic acid equivalent (GAE) / g dried extract, while the TFC ranged between 33.24 ± 3.23 and 135.85 ± 3.83 mg rutin equivalent (RUE) / g dried extract. The purified extract of *E. angustifolia* leaves contained higher TPC (203.76 ± 2.07 mg GAE / g dried extract) and TFC (135.85 ± 3.83 mg RUE / g dried extract). DPPH radicals scavenging capacity

and SPF of the tested extracts were positively correlated with their TPC and TFC. *Elaeagnus angustifolia* purified extract produced the lowest IC₅₀ value of 4.5 and exhibited higher SPF of 29.73 ± 0.31 as calculated for 2mg/ml methanol solution of the extract.

Conclusion: overall result of present study showed that *E. angustifolia* leaves purified extract was rich in phenolics and flavonoids, and showed high SPF value, hence could be highly recommended to be used in development of herbal sunscreen formulations.

11 **Keywords:** *Alhagi pseudalhagi*, *Elaeagnus angustifolia*, total phenolic content, total flavonoid content,
12 antioxidant, Sun protective factor

13 1. INTRODUCTION

14 Medicinal plants (MPs) have been used by mankind as curing agents since immemorial time, and still they are
15 known as natural remedies for divers array of maladies. According to WHO, about 80% of world population still
16 rely on traditional herbal medicines in case of their primary health care needs [1,2]. Herbal extracts owing to their
17 complex compositions exhibit different biological and pharmacological effects; such as anti-inflammatory,
18 antioxidant, antiapoptotic immunomodulatory, sun blocking, anti-cancer activities, and so on. Different botanical
19 extracts are reported for their free radical scavenging capacity, anti-photo aging effects, and their role in treatment of
20 different skin diseases and improvement of skin appearance [3-6].

21 Excessive solar UV radiations (UVR) that cause formation of excess Reactive Oxygen Species (ROS), are
22 known to be responsible for various skin damages such as; sunburn, skin pigmentation, premature aging, photo
23 carcinogenesis, and skin cancers [7-9].

24 Applying topical sunscreens is widely encouraged in order to protect skin from the adverse consequences of
25 solar UVR. An effective sunscreen besides containing active screening agents, should contain antioxidants to
26 scavenge ROS and to efficiently prevent skin from photo aging and skin cancers. Plants' antioxidants e.g. phenolic
27 compounds and flavonoids are suitable candidates to be incorporated in sunscreen formulations [10]. Currently,
28 plants as well as marine organisms are considered to be valuable sources of potential anti-photoaging and
29 photoprotective agents for development of cosmeceutical and topical pharmaceutical formulations [11].

30 *Alhagi pseudalhagi* (Syn. *A. maurorum*) belongs to family Fabaceae, is a small thorny shrub with stiff and spiny
31 stem and branches. The plant is called *shuthur khar* in Afghanistan and is used in traditional medicine of the country
32 for treatment of kidney disease, urinary tract infections, gonorrhoea, bleeding, wounds, hypotension, and

33 gastrointestinal disorders [12]. *Alhagi pseudalhagi* is reported to be rich in several bioactive compounds e.g.
34 flavonoids, flavone glycosides, alhagidin, alhagitin, proanthocyanidins, triterpenes, tannins, and phenolic compounds
35 [13, 14].

36 *Elaeagnus angustifolia* L. belongs to family Elaeagnaceae, is a small to medium sized tree or shrub, 2 – 5 m tall
37 and grow abundantly in different parts of Afghanistan [15]. *Elaeagnus angustifolia* is used in traditional medicines
38 as antipyretic, diuretic, tonic, antidiarrheal, anti-inflammatory, analgesic, and as a medication against kidney stones
39 [16]. Different parts of the plant including its leaves are rich in various phytochemicals such as; polysaccharides,
40 sitosterols, glycosides, terpenoids, coumarins, saponins, carotenoids, vitamins, tannins, phenolics and flavonoid
41 compounds that act as natural antioxidants [17-19].

42 Unfortunately, because of the long-lasting wars and other political issues, study of MPs grow in Afghanistan is
43 neglected, and hence, publications regarding phytochemical profile and biological activity of Afghani MPs are very
44 scarce. Considering worldwide interests into research on MPs and the huge demand of natural sunscreens, the main
45 objectives of present research were to evaluate the preliminary phytochemical profile, total phenolic content (TPC),
46 total flavonoid content (TFC), *in vitro* antioxidant potential, and sun protective factor (SPF) values of *A.*
47 *pseudalhagi* herb and *E. angustifolia* leaves collected from Afghanistan.

48 2. MATERIAL AND METHODS 49

50 2.1 Plant materials

51 *Elaeagnus angustifolia* leaves were collected from Paghman district of Kabul on 7th of June, 2017, and *A.*
52 *pseudalhagi* herb was collected at blooming time from Kabul University campus on 2nd July, 2017. Collected
53 materials were botanically authenticated by Prof. Mohammad Nasim sediqi, Head of Pharmacognosy department,
54 Faculty of Pharmacy, Kabul University. Collected materials were shade dried and after being coarsely powdered,
55 were separately packed in polyethylene bags for further use. Voucher specimens of the collected plants were
56 deposited in herbarium of faculty of Pharmacy, Kabul University, for further use as references.

57 2.2 Preparation of plants extracts

58 Conventional extraction method using soxhlet extractor was used in preparation of the crude extracts. 20 g of the
59 dried and powdered *A. pseudalhagi* herb and *E. angustifolia* leaves were separately extracted with 70% methanol

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60 (MeOH), at 70-°C, until the marc was exhausted. The extracts were filtered through Watmann No. 1, and then were
61 concentrated under vacuum, at 40-°C. Concentrated extracts were further dried in oven at 60-°C, until getting their
62 constant weights (complete drying). The dried *A. pseudalhagi* crude extract (APCE) and *E. angustifolia* crude
63 extract (EACE) were kept in refrigerator at 4-°C for further uses.

64 **2.3 Purification of extracts**

65 To purify the extracts from chlorophyll and other inert lipophilic compounds, the method described by Jarzycka *et*
66 *al.* with slight modifications were followed [20]. Accurately weighed amounts of both APCE and EACE were first
67 dissolved in 70% MeOH, then extracted 4 times with equal amounts of its volume petroleum ether. The MeOH
68 fractions were dried at 60 °C in drying oven. The dried extracts were then separately dissolved in hot distilled water,
69 followed by addition of ascorbic acid (0.5 mg/g dried extract) to the mixture. Each of the mixture was left in
70 refrigerator for 24 hours, and then, was extracted five times with diethyl ether. The last aqueous fractions were
71 further extracted five times with ethyl acetate. In both cases, the ethyl acetate fraction was evaporated to obtain the
72 dried *A. pseudalhagi* purified extract (APPE) and *E. angustifolia* purified extract (EAPE). APPE and EAPE were
73 used for further studies. The yield percentages of the crude and purified extracts were calculated and shown in
74 Table1.

75 **2.4 Preliminary phytochemical screening**

76 Methanol solutions (2 mg/ml MeOH 70%) of the extracts, e.g. APCE, EACE, APPE, and EAPE were separately
77 prepared and subjected to preliminary phytochemical screening to detect diverse categories of phytochemicals in the
78 extracts. Standard procedures and methods previously described were followed [21-23].

79 **2.4.1 Detection of carbohydrates**

80 **2.4.1.1 Molisch's test:** About 3 ml of test solution (TS) was mixed with 3 drops of Molisch's reagent (alpha-
81 naphthol 20% in ethyl alcohol) in a clean test-tube. Then about 2 ml of conc. H₂SO₄ was carefully added along the
82 side of the test-tube. Formation of a reddish violet or dark purple colour ring at the junction of the two layers
83 indicated presence of carbohydrates.

84 **2.4.1.2 Resorcinol test:** A few small crystals of resorcinol were dissolved in about 2 ml of TS in a test-tube. Then an
85 equal volume of conc. HCl was added to the test-tube, followed by warming on water-bath for a few minutes.
86 Formation of a rose colour indicates presences of ketoses.

87 **2.4.2 Detection of alkaloids**

88 **2.4.2.1 Dragendorff's test:** three drops of Dragendorff's reagent (potassium iodide + bismuth nitrate) were added on
89 2 ml of TS. Formation of an orange red/brown colour precipitate indicates presence of alkaloids.

90 **2.4.2.2 Hagers' test:** Few drops of Hagers' reagent (saturated picric acid solution) were added on 2 ml of TS.
91 Formation of a yellow precipitate reveals a positive result.

92 **2.4.2.3 Mayer's test:** Few (2 - 3) drops of Mayer's reagent (solution of Potassium mercuric iodide) were added on 2
93 ml of TS. Formation of a white, pale yellow or cream precipitate indicates presence of alkaloids.

94 **2.4.2.4 Wagner's test:** About 2 – 4 drops of Wagner's reagent (Iodine solution) were added on 2 ml of TS in a test-
95 tube. Formation of a brown or reddish-brown precipitate indicates a positive result.

96 **2.4.3 Detection of glycosides**

97 **2.4.3.1 Legal's test:** About 2 ml of TS were treated with 2 ml of sodium nitroprusside 2% solution in pyridine, and
98 few drops of 20% sodium hydroxide in MeOH. The mixture was observed for formation of a pink to deep blood red
99 colour.

100 **2.4.3.2 Keller-Kiliani test:** About 3 ml of TS were mixed with 2 ml glacial acetic acid and 1 ml ferric chloride (5%)
101 solution. The mixture was heated on water-bath, then cooled and transferred to a test-tube containing 2 ml of
102 concentrated H₂SO₄, and observed for formation of a reddish-brown colour at the junction of the liquids, and
103 gradually the acetic acid layer turns blue.

104 **2.4.4 Detection of anthraquinone glycosides**

105 **2.4.4.1 Borntrager test:** Few mg of the test extract was treated with 5 ml HCl 0.5 N, then it was mixed with 5 ml
106 chloroform for 15 minutes in a separatory funnel. Then, the mixture was placed aside for being separated into two
107 phases. The chloroform phase was taken in a clean test-tube, followed by addition of 2 ml of 10% ammonia
108 solution. The mixture was shaken well; then it was stand till the two phases were separated. Appearance of a pink
109 or reddish colour in the aqueous phase indicated a positive result.

110 **2.4.5 Detection of phenols**

111 **2.4.5.1 Ferric chloride test:** About 2 ml of TS was placed in a clean test-tube, followed by addition of 2 – 3 drops of
112 1% ferric chloride solution. Formation of an intense greenish-black colour indicates presence of phenolic
113 compounds.

114 **2.4.6 Detection of flavonoids**

115 | **2.4.6.1 Alkali reagent test:** About 2 ml of diluted TS was placed in a test-tube. A few drops of NaOH 1N solution
116 were added drop-wise in the test-tube. Increasing intensity of the yellow colour produced in the test-tube that would
117 be colorless by addition of few drops of dilute HCl, indicates presence of flavonoids.

118 **2.4.6.2 Shinoda/Pew test:** A small amount of magnesium turnings was added into a test-tube containing about 3 ml
119 of the TS. After addition of 3 drops of concentrated HCl in the test-tube, it was kept aside for completion of the
120 reaction. After a while it was observed for formation of a reddish-pink or rose colour for a positive result.

121 **2.4.7 Detection of tannins**

122 **2.4.7.1 Gelatin test:** About 2 ml of TS was treated with 2 ml aqueous solution of 1% gelatin containing 10% sodium
123 chloride. Formation of a white buff colour precipitate (a milky colour) indicates presence of tannins in the test
124 sample.

125 **2.4.8 Detection of saponins**

126 | **2.4.8.1 Foam test:** One ml of the TS after being diluted with 2 ml distilled water in a test-tube, was vigorously
127 shaken for 15 minutes. Formation of persistent foam reveals presence of saponins in the test sample.

128 **2.4.8.2 Fontan-kendale test:** To differentiate between triterpenic and steroidal saponins, 1 ml of the TS was placed
129 into two clean test-tubes (numbered 1 and 2), separately. Then, 5 ml of NaOH 0.1 N and 5 ml of HCl 0.1 N were
130 mixed with the contents of test-tube No. 1 and No. 2, respectively. Both of the test-tubes were simultaneously
131 shaken well, and were observed for the amount and stability of the foam produced in both test-tubes. Production of a
132 persistent foam in test-tube No. 1 (containing mixture of test sample and diluted alkali), than in test-tube No 2
133 (containing mixture of test sample and diluted HCl) indicates presence of steroidal saponins in the sample.

134 **2.4.9 Detection of sterols**

135 A small amount of the dried extract was washed with petroleum ether and then with acetone. The residue was
136 extracted with small amount (10 ml) of chloroform and the later was tested for detection of steroids.

137 **2.4.9.1 Liebermann – Burchard's test:** About 5 ml of the chloroform extract of the sample was placed in a
138 test-tube, followed by addition of few drops of acetic anhydride. Then 2 ml of conc. H₂SO₄ were carefully added
139 from the side of the test-tube. Formation of a brown to blood red colour at the junction of two phases indicates a
140 positive result.

141 **2.4.9.2 Salkowski Reaction:** About 5 ml of the chloroform extract was placed in a test-tube, followed by
142 carefully addition of about 2 ml of conc. H₂SO₄ from the side of test-tube. Formation of yellow-red or brown colour
143 ring at the junction, which turns red after a short time, indicates presence of sterols in the sample.

144 Results of phytochemical screening of the tested extracts are presented in Table 2.

145 **2.5 Determination of total polyphenolic content (TPC)**

146 Total phenolic content of APCE, APPE, EACE and EAPE was determined as per Folin-Ciocalteu
147 spectrophotometric method previously described [24, 25].

148 Briefly; 1 ml of extract MeOH solution having concentration of 300 µg/ml, was mixed with 1 ml Folin-
149 Ciocalteu's Reagent (FCR) diluted (1/10) with distilled water in a clean test-tube. The mixture was shaken well, and
150 placed at room ambience for 6 minutes, followed by addition of 3 ml Na₂CO₃ 7.5 % aqueous solution. Blank was
151 concurrently prepared as per the same procedure by substituting the extract with distilled water. Aliquots of gallic
152 acid (GA) at concentrations of 5, 10, 20, 30, 40, and 50 µg/ml in MeOH, were used for establishing a standard
153 calibration curve. All of the mixtures were shaken well, and incubated for 60 minutes at room ambience,
154 subsequently centrifuged at 3000 rpm for 10 minutes. The supernatants were checked for their absorbances at 765
155 nm wavelength against the blank, using a spectrophotometer (Shimadzu UVmini 1240). TPC of the tested samples
156 was calculated by use of the regression formula produced in GA calibration curve, and were expressed as mg GAE/g
157 dried extract, as shown in Table 3.

158 **2.6 Determination of total flavonoid content (TFC)**

159 The APCE, APPE, EACE, and EAPE were screened for their TFC values, applying slightly modified
160 Aluminum trichloride colorimetric method previously described [26, 27]. Briefly; AlCl₃ solution 2% (w/v) in MeOH
161 was freshly prepared as reagent solution. Two ml of sample extract MeOH solution (300 µg/ml) were well mixed
162 with 2 ml of the reagent in clean test-tubes. Two ml of Rutin (RU) aliquots at concentration range of 5 – 50 µg/ml
163 were used for establishing a standard calibration curve. All of the mixtures were shaken well, and incubated at room
164 ambience for 30 minutes in the dark for reaction completion. Then, absorbance of the mixtures was
165 spectrophotometrically measured at 415 nm wavelength against the blank sample consisting of the mixture of 2 ml
166 of sample/standard solution with 2 ml MeOH without AlCl₃. The TFC values were calculated based on the
167 regression formula produced from RU standard curve, and were expressed as mg RUE/g dried extract, as shown in
168 Table4.

169 2.7 Determination of *in vitro* antioxidant activity

170 For determination of *in vitro* antioxidant activity of tested extracts, DPPH assay method was used, as
171 previously described [26, 28]. Accurately, 4 ml of the test extracts serial dilutions in MeOH having concentrations
172 of 1.25, 2.5, 5, 10, 15, 20, and 25 µg/ml were separately mixed with 0.5 ml DPPH solution (0.5 mM) in MeOH.
173 Likewise, 4 ml of serial dilutions (0.25, 0.50, 0.75, 1.00, 1.5, and 2.0 µg/ml) of ascorbic acid in MeOH were mixed
174 with 0.5 ml DPPH solution (0.5 mM) in MeOH, to establish a standard calibration curve. As a positive control, 4 ml
175 of pure MeOH was mixed with 0.5 ml DPPH solution (0.5 mM). All of the resultant mixtures were thoroughly
176 shaken, and incubated in the dark at room temperature for 30 min for reaction completion. Then, absorbance of the
177 mixtures were screened at 517 nm wavelength against methanol as blank, using a UV-spectrophotometer (Shimadzu
178 UVmini 1240). The % inhibition for both of ascorbic acid and the tested extracts were calculated as shown in Fig. 1,
179 and Fig. 2.

180 2.8 Determination of Sun protective factor (SPF)

181 The SPF is most commonly used parameter for expression of sun protective capacity of sunscreens [10, 29].
182 SPF is UV energy required to produce a minimal erythema dose (MED) on protected skin, divided by the UV
183 energy required to produce a MED on unprotected skin. SPF is calculated using the following formula:

$$184 \text{ SPF} = \frac{\text{Minimal erythema dose in sunscreen protected skin}}{\text{Minimal erythema dose in nonsunscreen protected skin}}$$

185 MED is the lowest time interval or dosage of UV light irradiation, required to produce a minimal, perceptible
186 erythema or reddening on unprotected skin [30, 31].

187 SPF values of tested extracts were determined by *in vitro* spectrophotometric method based on measuring
188 transmittance of the extracts diluted solutions in MeOH. Transmittance of the extracts dilutions were measured in
189 the range of 292.5 to 337.5 nm, at five nanometers intervals. SPF values of extracts were calculated using the
190 following formula adopted from previous works [32, 33].

$$191 \text{ SPF} = \frac{\sum_{292.5}^{337.5} E(\lambda) \times \varepsilon(\lambda)}{\sum_{292.5}^{337.5} E(\lambda) \times \varepsilon(\lambda) \times T(\lambda)}$$

192 Where E (λ) is the spectral irradiance of terrestrial sunlight; and ε (λ) is the erythema action spectrum at λ, and
193 T (λ) is the measured transmittance at λ. The values of E (λ) and ε (λ) are constant, and derived from previous
194 works. To establish a standard calibration curve, SPF values of five different concentrations of the test extracts
195 were measured and the related graph was plotted.... SPF values for tested extracts MeOH solution (2 mg/ml)

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196 were calculated using regression formula obtained from the plotted standard curve, and the results are shown in
197 Table 5.

198 2.9 Statistical Analysis

199 All of the experiments were performed in triplicates (n=3) and values were expressed as Mean±SD. Statistical
200 analyses were performed using Ms. Excel 2016 package. One-way analysis of variance (ANOVA) and Student t-test
201 were used to assess the differences between different variables. All analyses were performed at the 5% significant
202 level (p<0.05).

203 3. RESULTS AND DISCUSSION

204

205 3.1 Yield percentages of the extracts

206 The yield percentage of the extracts for *A. pseudalhagi* herb and *E. angustifolia* leaves are presented in (Table 1).

207 Table 1. Data showing the extractive % (w/w) of the plants

S. No.	Extract type	% extractive Mean±SD*
1	APCE	31.834±1.64
2	EACE	35.51±1.62
3	APPE	5.308±0.10
4	EAPE	4.475±0.08

208 * Mean ± Standard Deviation of the three experiments (n=3),

209 APCE; *A. pseudalhagi* herb crude extract, APPE; *A. pseudalhagi* processed extract,

210 EACE; *E. angustifolia* leaves crude extract, EAPE; *E. angustifolia* processed extract

211 As shown in Table 1, the highest yield is recorded for EACE (35.51±1.62 %), followed by APCE (31.834±1.64 %),
212 while yields % for APPE and EAPE were recorded to be 5.308±0.10 and 4.475±0.08, respectively.

213 As data show, the percentage yields of the purified extracts are lower than that of the crude extracts, that
214 indicates most of extra materials such as chlorophyll and other lipophilic substances are removed during washing of
215 the crude extract by petroleum ether. Such purified extracts are proved to pose more potent biological efficacy
216 because of removal of the inert compounds thereof.

217 3.2 Phytochemical screening of the extracts

218 Phytochemicals present in the tested extracts are shown in Table 2.

219 Table 2. Phytochemicals detected in the tested extracts

S. No.	Phytochemicals	APCE	APPE	EACE	EAPE
--------	----------------	------	------	------	------

01	Carbohydrates				
	Molisch's test	++	+	++	+
	Resorcinol test	+++	+	++	+
02	Phenols				
	FeCl ₃ Test	++	+++	++	+++
03	Flavonoids				
	Alkali R. Test	+	+++	++	+
	Shinoda Test	+	++	++	+
04	Tannin				
	Gelatin test	++	-	++	-
05	Saponin				
	Foam test	+	++	+	-
	Fontan-Keldale test	triterpenic	triterpenic	steroidic	-
06	Sterols				
	Liebermann – B test	+	+	+	+
	Salkowski test	+	+	+	+

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220 APCE; *A. pseudalhari* herb crude extract, APPE; *A. pseudalhari* processed extract, EACE; *E. angustifolia* leaves crude
 221 extract, EAPE; *E. angustifolia* processed extract, '+' Present, '++' moderately present, '+++' intensely present, '-' absent

222 As shown in Table 2, different classes of phytochemicals such as; carbohydrates, flavonoids, phenolic compounds
 223 and saponins were found to be present in all tested extracts. Based on qualitative phytochemical tests, *A.*
 224 *pseudalhari* herb contains triterpenic saponins while *E. angustifolia* leaves contain steroidal saponins. However,
 225 further works and quantitative tests are required for accurate determination of the phytochemicals present in the
 226 tested extracts. Based on available literature, presence of diverse array of secondary metabolites is reported in
 227 *Alhagi* species including *A. pseudalhari* [14, 34, 35]. Similarly, *E. angustifolia* L. is reported to contain different
 228 compounds such as; alkaloids, flavonoids, polysaccharides, saponins, tannins, vitamins, and metal elements in
 229 different plant parts [17, 18, 36, 37].

230 3.3 Total phenolic content (TPC) of the extracts

231 In present study, extracts MeOH solution at (300 µg/ml) were screened for their TPC values, applying
 232 Folin-Ciocalteu method previously described [25, 38]. TPC of tested samples were calculated from the regression
 233 equation ($y=0.0201x + 0.024$, $R^2=0.995$) obtained from GA standard curve, and were expressed in terms of GA
 234 equivalent (mg of GAE/g of dried extract), as shown in Table 3.

235 **Table 3.** Data showing the TPC values of tested extracts

S. No.	Extract type	TPC values *(mg GAE/g dried extract)
1	APCE	71.14±3.10
2	APPE	80.43±3.69
3	EACE	82.64±2.57
4	EAPE	203.76±2.07

236 * The values are recorded as Mean±SD, (n=3)

237 APCE; Alhagi pseudalhagi crude extract, APPE; A. pseudalhagi processed extract, EACE; Elaeagnus angustifolia
238 crude extract, EAPE; E. angustifolia processed extract

239 As shown in Table 3, the order of TPC in tested extracts was highest for EAPE > EACE > APPE > APCE.
240 Consequently, among tested extracts, both of the EAPE and EACE exhibited highest TPC values of 203.76±2.07
241 and 82.64±2.57 mg GAE/g dry extract, respectively. The data showed that purified extracts revealed higher TPC
242 values than that of crude extracts. Plants phenols, by virtue of their aromatic rings, show an intense absorption in the
243 UV region of the spectrum [22]. Hence, they would serve as choice compounds to be incorporated in sunscreen
244 formulations.

245 3.4 Total flavonoid content (TFC) of the extracts

246 Determination of TFC of botanicals is of high importance in predicting biological activities, such as
247 antioxidative potential of tested crude drugs. In present study, TFC of tested extracts were calculated from the
248 regression equation ($y=0.023x - 0.072$, $R^2=0.9996$) obtained from RU standard curve. The TFC values were
249 expressed in terms of rutin equivalent (mg of RUE/g of dried extract), and shown in Table 4.

250 **Table 4.** Data showing TFC values of tested extracts

S. No.	Extract type	TFC values *(mg RUE/g dried extract)
1	APCE	59.61±3.40
2	APPE	121.06±3.37
3	EACE	33.24±3.23
4	EAPE	135.85±3.83

251 * The values are recorded as Mean±SD, (n=3)

252 APCE; Alhagi pseudalhagi crude extract, APPE; A. pseudalhagi processed extract, EACE; Elaeagnus
253 angustifolia crude extract, EAPE; E. angustifolia processed extract

254 As shown in Table 4, the order of TFC of tested extracts was highest for EAPE > APPE > APCE > EACE,
255 respectively. TFC of the tested extracts varied from 33.24±3.23 to 135.85±3.83 mg RUE/g dried extract. The highest
256 flavonoids content (135.85±3.83 mg RUE/g extract) was recorded for EAPE.

257 3.5 *In vitro* antioxidant activity of the plant extracts

258 Evaluation of antioxidative potential of botanicals serves as a choice approach to judge their
259 pharmaceutical as well as pharmacological values. In present work, sample extracts at concentration range of 2.5 –
260 25 µg/ml in MeOH were evaluated for their DPPH free radical scavenging activity. Ascorbic acid serial dilutions at
261 concentration range of 0.25 – 2 µg/ml in MeOH were used as standard antioxidant compound. Radical scavenging
262 activity (RSA) of the test extracts as well as standard ascorbic acid was calculated as percentage inhibition (%
263 inhibition) of DPPH free radicals, applying the following formula:

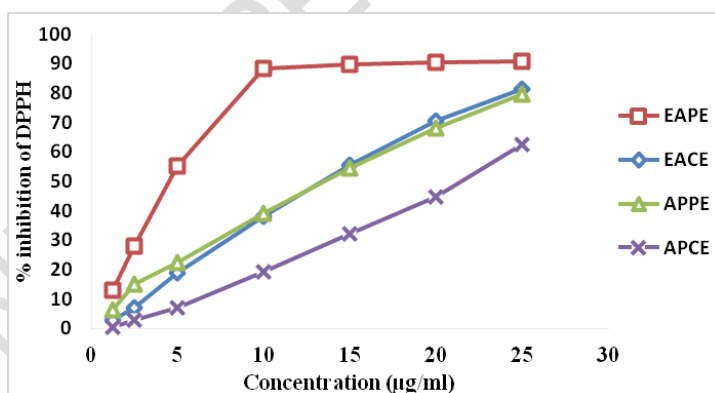
$$264 \quad \% \text{ inhibition} = 100 \times [(Ac - As) / Ac]$$

265 Where, **Ac** is the absorbance of control, and **As** is absorbance of sample/standard

266 Antioxidative potential of the standard ascorbic acid and tested extracts were expressed as inhibition
267 concentration of 50% DPPH free radicals (IC₅₀), which were calculated from their plotted graph of % inhibition.

268 Figure- 1, represents the % inhibition of DPPH free radicals by the tested extracts.

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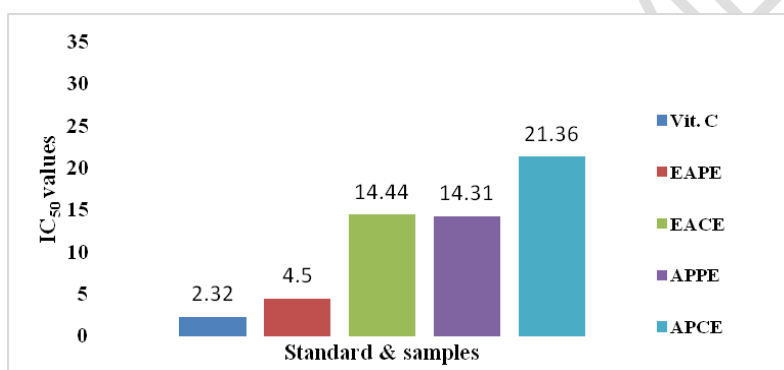


269
270 **Fig. 1.** Graph showing % inhibition activity of tested extracts in DPPH assay: EAPE; *E. angustifolia* processed
271 extract, EACE; *E. angustifolia* leaves crude extract, APPE; *A. pseudalhagi* processed extract, and APCE; *A.*
272 *pseudalhagi* herb crude extract

273 As shown in Fig. 1, the RSA of the tested samples was dose-dependent. That is to say, the % inhibition of
274 DPPH free radicals were increasing by increase in concentration of test extracts solutions used in the assay.

275 The antioxidant potentials of standard ascorbic acid and tested extracts were expressed as their IC₅₀ values.
276 IC₅₀ actually refers to the concentration (µg/ml) of standard or sample extracts required for inhibition of 50% DPPH
277 radicals used in the assay. Lower IC₅₀ value of a test sample indicates higher RSA and higher antioxidant activity of
278 the same sample [26, 27, 39].

279 *In vitro* antioxidative potential of tested extracts were expressed as their IC₅₀ values which were calculated
280 from their plotted graphs of % inhibition vs. concentrations (Fig. 1.). Fig. 2 represents the calculated IC₅₀ values for
281 both ascorbic acid and the tested extracts.



282
283 **Fig. 2.** Graph showing the IC₅₀ values for the standard and tested extracts: *Vit. C*; vitamin C, *EAPE*; *E.*
284 *angustifolia* processed extract, *EACE*; *E. angustifolia* leaves crude extract, *APPE*; *A. pseudalhagi* processed
285 extract, and *APCE*; *A. pseudalhagi* herb crude extract

286 As shown in Fig. 2, IC₅₀ for ascorbic acid is 2.32 µg/ml, while IC₅₀ of the tested extracts are in range of 4.5 – 21.36
287 µg/ml. EAPE exerts the highest free RSA (IC₅₀ = 4.5), while RSA of the remaining extracts decreased in order of
288 APPE>EACE>APCE with IC₅₀ values of 14.31<14.36<21.36, orderly.

289 In DPPH assay, the stronger activity of EAPE could be attributed to its purity from other inert
290 phytochemicals such as chlorophyll, etc. and its richness in phenolics compounds. Therefore, a direct correlation
291 exists between TPC and DPPH radical scavenging potential of EAPE. Our data were in compliance with those of
292 many previous works which reported positive correlation between DPPH radical scavenging activities and TPC
293 values of tested plant extracts [26, 40]. As per another study, high phenolic content and strong antioxidant properties
294 of ethylacetate fraction of defatted methanolic extract of *Allium* species was reported [41]. In another study,

295 methanol extracts of cinnamon and cloves exhibited strong antioxidant properties owing to their high phenolic and
296 flavonoids content [38]. Positive correlation between TPC and in vitro antioxidant potential of *Aloe vera* leaves is
297 also reported [42].

298 3.6 Sun protective factor (SPF) of the extracts

299 SPF values of the tested extracts' MeOH solution (2mg/ml) are presented in Table 5.

300 **Table 5.** Data showing SPF value for the tested extracts 2mg/ml methanolic solutions

S. No.	Extract type	SPF* (Conc. 2 mg/ml)
1	APCE	5.47 ± 0.03
2	APPE	17.83 ± 0.88
3	EACE	10.69 ± 0.16
4	EAPE	29.73 ± 0.31

301 * Data were recorded as Mean±SD, (n=3).

302 APCE; *Alhagi pseudalhagi* crude extract, APPE; *A. pseudalhagi* processed

303 As per the results, the SPF values were between 5.47 ± 0.03 and 29.73 ± 0.3. As data showed, both of EAPE
304 and APPE exhibited higher SPF than EACE and APCE. This issue could be attributed to the purity of EAPE and
305 APPE from excess of chlorophyll and other inert compounds. The higher amounts of both polyphenols and
306 flavonoid compounds in EAPE and APPE, and elimination of their inert chemicals, are claimed to be the reasons for
307 their increased UV absorption.

308 The *In vitro* spectrophotometric method used in present study, was based on measuring transmittance of
309 diluted solution of the tested extracts. Since botanical extracts contain a wide range of phytochemicals, such as;
310 phenolic compounds, flavonoids, carotenoids, vitamin E, vitamin C and etc., hence these compounds are responsible
311 for UV absorption and sun protective properties of botanicals [10, 29, 43].

312 In present study, positive correlations were found between DPPH radical scavenging capacity and SPF values of
313 the tested extracts. Likewise, there was positive correlation between SPF and TPC, and also between SPF and TFC
314 of the tested extracts. Our findings were in compliance with many previous works, which have reported that both
315 phenolics and flavonoid compounds act as UV absorbing agents [10, 32, 44]. In addition, phenolics and flavonoids
316 as strong antioxidant agents, play important roles in prevention of UV induced damage such as; photo aging and
317 skin cancers [20, 45]. Recently, antioxidants present in botanicals and marine organisms are deemed to serve as
318 compounds of choice for developing natural sunscreen formulations [10, 11].

Comment [Z6]: It should be useful to present (in a table) the obtained values of the correlation coefficients.

319 **4. CONCLUSION**

320 Considering the huge demand for safe and effective plants derived UV-filters, *A. pseudalhagi* herb and *E.*
321 *angustifolia* leaves were screened for their antioxidant potential and sun protective factor. Purified extract of *E.*
322 *angustifolia* leaves being rich in phenolics and flavonoids, demonstrated strong DPPH radicle scavenging activity
323 and a high SPF of 29.73 ± 0.31 for its MeOH solution (2mg/ml). Hopefully, *E. angustifolia* leaves could be
324 recommended as natural source of the sun protective agents beneficial in developing potent herbal sunscreen
325 formulations.

326

327 **CONSENT**

328 Not applicable.

329 **ETHICAL APPROVAL**

330 Not applicable.

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